



UNIVERSITY
OF TRENTO

PhD Program in Biomolecular Sciences

Department of Cellular, Computational
and Integrative Biology - CIBIO

37th Cycle

Modulation of BAZ2 Proteins as a Potential Therapeutic Strategy in Cancer and Neurological Disorders

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Academic Year 2023-2024



UNIONE EUROPEA
Fondo Sociale Europeo





Università degli Studi di Trento

Dipartimento di Cellular, Computational and Integrative Biology

Dottorato di Ricerca in Scienze Biomolecolari

37^{esimo} Ciclo

Tesi di Dottorato

*Modulation of BAZ2 Proteins as a Potential Therapeutic Strategy
in Cancer and Neurological Disorders*

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Dottoranda

Eleonora Parolin

anno accademico 2023-2024

La borsa di dottorato è stata cofinanziata con risorse del
Programma Operativo Nazionale Ricerca e Innovazione 2014-2020 (CCI 2014IT16M2OP005)
Fondo Sociale Europeo, Azione I.1 "Dottorati Innovativi con caratterizzazione Industriale"

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Modulation of BAZ2 Proteins as a Potential Therapeutic Strategy in Cancer and Neurological Disorders

Eleonora Parolin, 37th Cycle

March 20, 2025

Abstract

The complex landscape behind protein homeostasis involves a highly sophisticated system devoted to maintaining cellular balance and preventing dysfunction. In this context, the cellular machinery acts as a quality control mechanism, rapidly identifying and eliminating proteins that could impair normal cell functioning. Currently, such a mechanism is strategically employed by researchers to promote the degradation of proteins of interest (POI), acting as relevant players in pathological conditions. The cellular degradation system is "hijacked" toward target elimination by exploiting PROteolysis Targeting Chimera (PROTAC) technology. This innovative approach allows protein degradation by fostering interactions between an E3 ubiquitin ligase and a POI, effectively directing the ubiquitin-proteasome system to eliminate the target protein.

In this study, we harness the potential of the PROTAC technology to promote the degradation of BAZ2A protein while providing insights into the complex mechanism relating this target to prostate cancer. Recent studies have highlighted BAZ2A's role in late-stage prostate cancer, prompting us to explore its potential as a therapeutic target. Therefore, we first interfered with BAZ2A pathological functions by hampering through a competitive inhibitor its ability to recognize acetylated lysines (Kac) at the histone tails. Although Kac recognition represents a key BAZ2A function, simple inhibition did not produce any antiproliferative effect in prostate cancer cell lines. On the other hand, BAZ2A ablation through RNA interference displayed promising results by slowing the proliferation rate and strongly affecting the expression of genes related to BAZ2A dysregulation in cancer. Thus, we decided to exploit PROTAC technology to replicate the beneficial effect observed upon BAZ2A ablation through silencing RNAs. BAZ2A binding PROTAC molecules were designed based on available BAZ2A structures. The synthesis of eleven BAZ2A degraders was accomplished, categorizing PROTACs according to their capability to recruit CRBN or VHL E3 ligases. Preliminary experiments revealed that VHL-based degraders featuring longer linkers promoted BAZ2A degradation, confirming the PROTAC potential in successfully mediating POI degradation. Attempts at X-ray structure determination of ternary complexes of BAZ2A/B, a PROTAC molecule and VHL-EloB/C resulted in structures of binary complexes at best and will form the basis of future structural efforts towards the same targets. Here, we report an extensive study on the role of BAZ2A in tumorigenic conditions, proposing targeted protein degradation as a promising strategy to counteract aggressive prostate cancer. Notably, BAZ2A ablation was achieved by successfully designing and synthesizing PROTAC degraders. Future work will confirm the therapeutic potential of this strategy, including the possibility of targeting BAZ2B, a related protein implicated in the ageing process.

DECLARATION

I, ELEONORA PAROLIN, CONFIRM THAT THIS IS MY OWN WORK AND THE USE OF ALL MATERIAL FROM OTHER SOURCES HAS BEEN PROPERLY AND FULLY ACKNOWLEDGED.

Signature

Eleonora Parolin

THE CONTENTS PRESENTED IN THIS THESIS ARE THE RESULT OF AN INTERDISCIPLINARY STUDY THAT INVOLVED THE EFFORT OF EXPERIMENTAL SCIENTISTS FROM DIFFERENT INSTITUTIONS. MY SPECIFIC CONTRIBUTIONS AS WELL AS THAT OF THE OTHER RESEARCHERS COLLABORATING IN THIS WORK ARE DESCRIBED IN DETAIL IN THE AUTHOR CONTRIBUTIONS SECTION.

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Chapter 1

Introduction

1.1 Protein Degradation

1.1.1 PROTAC Technology: Redefining the Future of Drug Discovery

The field of drug discovery has experienced significant changes over the past few decades. The emergence of biologics and significant advances in small molecule discovery have transformed the drug market over the past two decades, consistently driving revenue growth^[1]. However, a completely new pharmacological strategy emerged recently and shifted the classical paradigms unprecedentedly. PROteolysis Targeting Chimeras (PROTACs) exploit the endogenous cellular degradation machinery to modulate target protein expression through the ubiquitin-proteasome system (UPS). Such technology takes advantage of a heterobifunctional molecule composed of a protein of interest (POI) ligand and an E3 ubiquitin ligase (E3) recruiting moiety bridged by a linker. In this way, PROTAC molecules foster the interaction between the POI and E3, promoting target polyubiquitination and subsequent degradation through the 26S proteasome (Figure 1.1)^[2].

PROTAC technology relies on an innovative mode of action (MoA) based on the transient formation of ternary complexes to induce POI elimination, known as event-driven. Once the target protein is polyubiquitinated, PROTAC molecule is recycled and mediates the contact between E3 and another POI. Differently, classical small molecule inhibitors act in an occupancy-driven manner. Traditional inhibition functions by targeting binding pockets on the POI, thereby disrupting its activity. However, this mechanism has several drawbacks, including the need for stoichiometric engagement, high target affinity, and continuous administration^{[3][4]}.

On the contrary, the same PROTAC molecule can promote the formation of more than one ternary complex, thus requiring a lower dosage and a reduced recurrent administration. As a consequence, degraders could decrease the risk of side effects. In addition, degraders MoA requests only transient interaction with the target to promote POI elimination. Thus, PROTACs require a short-lived formation of the ternary complex without needing a constant occupation of the binding pocket. In this way, degraders can address two main problems in drug discovery: targeting the "undruggable" and overcoming resistance^[5].

In the classical drug discovery paradigm, targets should present a well-defined pocket, a deep cavity capable of accommodating a small molecule regulator. Approximately 80% of disease-related proteins are considered "undruggable" due to a flat surface and the lack of a cavity. Thanks to the weaker target affinity requested, PROTAC technology can investigate the vast majority of targets currently unexplored^[6]. For example, Wang's group designed a degrader molecule for STAT3. The latter acts as a signal transducer and activator, belonging to the broader family of STAT proteins. STAT3 overexpression relates to the occurrence and develop-

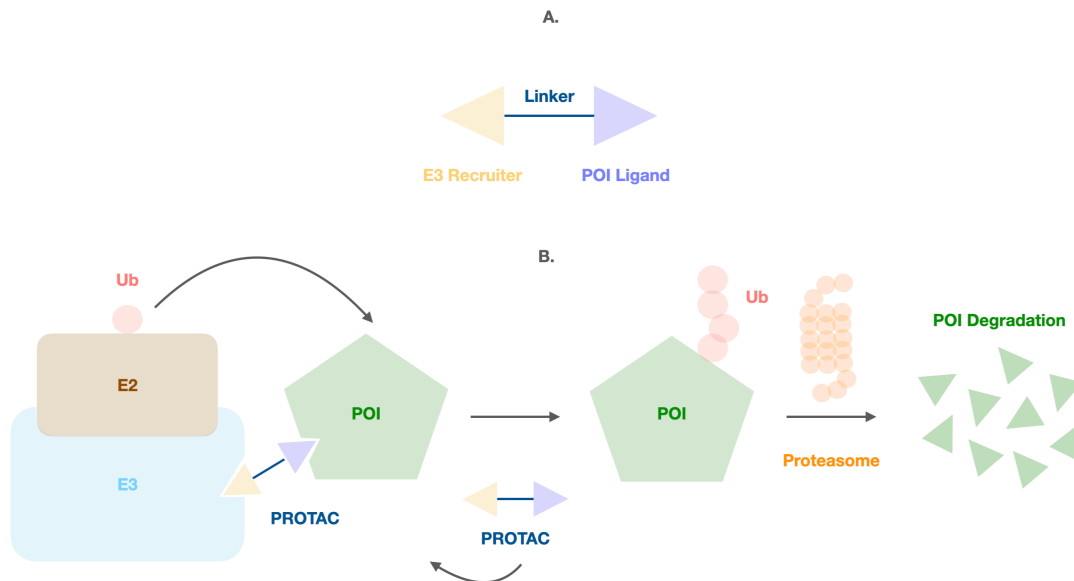


Figure 1.1: PROTAC's Mechanism of Action - Panel **A** illustrates the PROTAC structure formed by the ligand for a POI, an E3 ubiquitin ligase recruiter and a linker. Panel **B** shows PROTACs MoA: degraders force contact between a target protein and E3 ubiquitin ligase, which recruits an E2 ubiquitin-conjugating enzyme and mediates POI ubiquitination. Once the POI is polyubiquitinated and tagged for degradation, the 26S proteasome degrades it. During the process, PROTAC is recycled and mediates the formation of other ternary complexes, as shown at the bottom of panel **B**. Figure adapted from Li & Crews^[3].

ment of cancer, highlighting its possible role as a new tumoral target. However, STAT3 presents a convex positively charged structure at the level of its DNA-binding domain. At the same time, the Src homology 2 (SH2) domain is widely conserved and difficult to target specifically^[7]. STAT3 remained a "hard-to-target" protein until the development of the first PROTAC. The degrader, conceived by exploiting a known STAT3 ligand, efficiently eliminates the target and induces tumour regression in xenograft models^[8]. As reported by Chessum^[9], degraders can serve as a powerful tool for understanding the cellular role of proteins like Pirin. The latter is an iron-binding protein that works as a transcription regulator by interacting with antiapoptotic transcription factor NF- κ B and B-cell lymphoma 3 coding protein (BCL-3) and promoting their binding to DNA. Recent studies showed that Pirin imbalance could lead to cell proliferation and tumorigenesis, but the molecular mechanisms behind its pathological function were still unclear^{[10][11]}. In addition, Pirin's enzymatic function or endogenous ligands were not known. In this context, Pirin degraders were fundamental to discover the first Pirin ligand and to better investigate its function^[12].

As anticipated, PROTAC technology is a viable approach for overcoming drug resistance^{[3][4][5]}. The development of resistance to treatment has become one of the most significant challenges in clinical practice. Mutations at the level of the target protein commonly occur in cancer and infectious diseases, leading to a reduced or abolished response to drug treatment^[13]. Thus, the

transient fashion of PROTAC technology, requiring a reduced target affinity, resulted in less susceptibility to this resistance mechanism. For instance, chronic lymphocytic leukaemia patients are prone to develop drug resistance when treated with BTK covalent inhibitors, while BTK degraders efficiently enable target elimination in both wild-type and mutated forms^[14]. In addition, PROTAC technology successfully eludes resistance mechanisms arising from other determinants. Androgen receptor (AR) mutations commonly emerge upon Enzalutamide treatment. Frequently, target inhibition leads to AR overactivation, converting Enzalutamide into an AR agonist. However, the evolution of AR inhibitor into degrader solved the acquired target resistance, promoting target degradation independently from mutated AR forms^[15]. Three main factors are proposed to explain PROTACs' ability to overcome resistance: their effectiveness in counteracting feedback mechanisms related to cellular protein homeostasis, the requirement for only mild target affinity, and their sub-stoichiometric mode of action^[5].

However, PROTAC technology goes beyond overcoming drug resistance and targeting undruggable proteome. Through an innovative MoA, these bivalent degraders tackle the major challenge of reducing off-targets by enhancing selectivity and specificity. On-point structural modifications can induce selective POI degradation by exclusively promoting the formation of conformationally favoured ternary complexes, avoiding the elimination of additional proteins by blocking their ability to recruit simultaneously other targets^{[3][4][5]}. For instance, cyclin-dependent kinases (CDKs) work as serine-threonine kinases and exert a wide range of functions involving cell cycle regulation and modulation of gene expression. CDK4 and CDK6 belong to this broad family and are found dysregulated in different cancers. Palbociclib, which acts as CDK4/6 inhibitor, is recurrently administered in the treatment of breast cancers. However, specific CDK4 or CDK6 inhibitors are currently not available. Despite CDK4 and CDK6 having almost identical structures, CDK6 shows a different distribution of exposed lysines. Thanks to this observation, scientists hypothesized that palbociclib-based PROTAC could eventually degrade only CDK6. As expected, this degraders class eliminate efficiently CDK6 without influencing the expression of CDK4^[16]. Another interesting example showing PROTACs' ability to increase specificity was published by the Crews group^[17]. In the article, they exploited a promiscuous kinase inhibitor potentially interacting with more than 100 kinases and attached it to E3 ligase recruiting moiety. Upon PROTAC generation, only a few kinases were degraded, widely restricting the number of involved targets previously observed in classical inhibition. Reasons behind the drastic increase in specificity are found in the innovative PROTAC MoA that includes the contribution of important protein-protein interactions (PPI) occurring between the POI and the E3 ubiquitin ligase. In other words, cooperative binding involving proteic components strongly influences the formation of active ternary complexes, selecting only POI substrates positively interacting with the E3 ligase^[17].

In addition, PROTAC technology holds immense promise for revolutionizing cancer therapy. Ablation of tumour-related proteins represents a valid therapeutic approach to promote cancer regression. Over the years, various strategies have been explored, including gene editing procedures such as the CRISPR-Cas9 system and RNA interference^{[18][19]}. However, all the proposed approaches have proven to be expensive in terms of time and cost. In this context, PROTAC technology offers a potential method to rapidly and reversibly knock down or knock out POI, which genome editing strategies cannot accomplish^[5]. For example, the first PROTAC to reach clinical phase III effectively degrades estrogen receptor (ER), a common target in breast cancer therapy. This groundbreaking PROTAC molecule could offer a promising novel strategy for treating ER+/HER2- breast cancer^[20].

Lastly, traditional inhibition methods are ineffective in addressing non-enzymatic functions; PROTACs can easily target proteins holding kinase-independent scaffolding roles^[5]. For example, Focal Adhesion Kinase (FAK) is a well-known target in cancer, presenting enzyme-linked and

scaffolding functions. The group of Yu Rao reported the development of a potent focal adhesion kinase (FAK)^[21]. Thus, PROTAC technology enables exploring non-related enzyme functions besides their powerful role as therapeutic agents.

Since the first PROTAC was envisioned in 2001^[2], the field has witnessed numerous milestones, culminating in the first degraders successfully reaching clinical phase III trials. Despite these advancements, several challenges remain, offering an opportunity for innovation and growth. A primary concern is the unfavourable physicochemical properties of PROTACs; several developed degraders exceed Lipinski's "rule of five", which determines drug-likeness for orally administered drugs^{[3][4]}.

An essential component of the PROTAC chemical structure is the linker moiety, whose design is crucial for effective target degradation. Typically, linkers are alkyl or PEG-like linear chains, making them more susceptible to oxidative metabolism and rapid excretion. In general, PROTAC design poses significant challenges from a medicinal chemistry perspective; the site of connection and linker length greatly influence PROTAC functionality. Although several advancements have been made in optimizing linker structures, there are no established design principles yet, and the primary strategy remains trial and error based^{[5][22]}.

The key event for PROTAC technology is represented by the assembly of ternary complexes, which then determine a series of events culminating in POI degradation^[23]. Capturing a snapshot of ternary complex formation is crucial for understanding the MoA of degraders. Currently, only a few structures have been determined and published, all obtained through X-ray crystallography and cryogenic electron microscopy (Cryo-EM)^{[23][24][25]}. Isothermal Titration Calorimetry (ITC), AlphaLISA, Surface Plasmon Resonance (SPR), and Time-Resolved Fluorescence Energy Transfer (TR-FRET) were also exploited to better characterized ternary complex formation^{[23][26][27]}. While various strategies and protocols exist for characterizing ternary complex formation, substantial work remains to investigate these critical aspects of PROTAC technology better^[28].

The efficiency of POI degradation is heavily dependent on PROTAC concentration. Although higher doses typically enhance target elimination, excessively high concentrations can induce the "hook-effect". This phenomenon occurs when inactive binary complexes form, where the E3 ligase and POI are recruited separately, potentially leading to unwanted effects. Such event is unpredictable and target-specific, thus requiring considerable effort to adjust the correct concentration and elude the "hook effect"^[5].

One of the major concerns in the PROTAC field is related to the E3 ligase recruiting moiety. Scientists speculated that more than 600 E3 ubiquitin ligases are expressed in humans, however, only a small fraction of these were exploited to mediate target protein degradation^[29]. The most commonly recruited E3 ligases include Cereblon (CRBN), Von Hippel Lindau (VHL), Mouse Double Minute 2 Homolog (MDM2), and Cellular Inhibitor of Apoptosis Protein 1 (cIAP1). In this context, CRBN and VHL E3 ligases are preferentially used in PROTAC development. Both proteins belong to the large Cullin-RING ligases (CRLs) family, which is directly involved in the ubiquitination and subsequent degradation of cellular proteins. All CRL members present a recurrent architecture, holding a Cullin protein (CUL) bridging a substrate receptor (like CRBN or VHL, for example) to a RING domain-containing RBX-family protein. CUL activation is controlled by a neddylation process, involving the interaction between a CUL subunit and NEDD8^[30]. As previously mentioned, CRBN and VHL degradation takes place by engaging different molecular players. Degradation mediated by CRBN occurs through the involvement of damaged DNA binding protein 1 (DDB1), CUL4A, and regulators of cullin (ROC1)^[31], while VHL cooperates with Elongin B (EloB), Elongin C (EloC) and CUL2^[32]. In physiological conditions, CRBN mediates the elimination of MEIS2, ILF2 or GLUL^{[33][34]}, whereas VHL mainly provides for hypoxia-inducible factor 1a (HIF1a) degradation^[32]. VHL and CRBN are only two

of approximately 300 CRL members, highlighting the unexplored potential of remaining E3 ligases in targeted protein degradation^{[29][35]}.

In conclusion, PROTAC technology offers a groundbreaking approach to induce POI degradation, unlocking new therapeutic possibilities and shedding light on previously unexplored protein functions. The aforementioned disadvantages need to be considered, but the first PROTAC molecule reaching clinical phase III proves that major concerns can be successfully overcome. This vibrant and rapidly evolving field has already made remarkable progress, and the future promises even more exciting breakthroughs.

1.1.2 Targeted Protein Degradation beyond PROTAC Technology: Mechanisms of Action and Applications

Since the development of the first PROTAC, the field of protein degradation has witnessed groundbreaking advancements, revolutionizing our ability to induce protein degradation. This powerful tool redefined how we approach targeted degradation and paved the way for a diverse array of technologies aimed at precisely modulating protein elimination.

Another valuable technology exploiting the UPS pathway is molecular glues. The latter work in an event-driven manner and facilitates stable interactions between an E3 ligase and a protein substrate (Figure 1.2)^[36]. Molecular Glues facilitate protein-protein interactions, typically by stabilizing a transient interaction between a target protein and an E3 ubiquitin ligase, leading to ubiquitination and subsequent degradation. They work by enhancing natural binding interfaces rather than bridging two proteins with a linker. They are usually small, single-component molecules that optimize existing binding interfaces; they rely on stabilizing an existing weak or transient interaction, making their discovery more serendipitous and often limited to certain targets^[37]. Most known glues are thalidomide-based immunomodulatory imide drugs (IMiDs), whose protein degradation capabilities were discovered accidentally. Such compounds take advantage of CRBN E3 ubiquitin ligase complex to mediate the elimination of multiple substrates. Thalidomide-like compounds show an increased affinity for transcription factors holding zinc finger domains, like Ikaros family zinc finger protein 1 (IKZF1) and 3 (IKZF3)^[36]. In addition, this class of compounds promotes the degradation of casein kinase 1 α (CK1 α), a well-known target in myelodysplastic syndrome^[38]. Thalidomide analogues present a different solvent-exposed surface when bound to CRBN, defining target selectivity in this way^[39]. CRBN recruiting glues also include compound CC-90009, which induces GSPT1 (G1 to S phase transition 1) degradation, and it is under evaluation for acute myeloid leukaemia (AML) treatment^[40]. Also, the anticancer drug Indisulam acts as molecular glue and enables RNA binding motif protein 39 (RBM39) elimination by hijacking CUL4-DCAF15 E3 ligase^[41].

In general, molecular glues efficiently promote protein degradation while maintaining advantageous structural features. However, their discovery is currently not predictable, limiting the possibility of enlarging the array of targets^[42].

Proteasome degradation accounts for the great majority of intracellular protein elimination, while membrane-bound and secreted proteins mostly undergo degradation via the lysosomal pathway. The latter leverages cell surface receptors, such as CI-M6PR, to recognize substances containing specific motifs and initiate a degradation cascade. Specifically, biomolecules are internalized inside the cell through endocytosis and then incorporated into lysosomes to be eliminated^[49]. Inspired by this mechanism, Bertozzi's group developed the first lysosome-targeting chimeras (LYTACs). By conjugating a CI-M6PR ligand to a small molecule or antibody that binds a protein of interest (POI), they successfully promoted the degradation of extracellular targets (Figure 1.2)^[45]. One of the most relevant studies uses LYTAC to degrade epidermal growth factor (EGFR), whose elimination presented an increased anti-proliferative effect on HepG2 cells compared to the POI ligand alone. Notably, EGFR ablation was maintained for at least 72 hours upon LYTAC treatment, and no "hook effect" was detected. Other proteins, including programmed death-ligand 1 (PD-L1), apolipoprotein E4 (ApoE4), and CD71, have also been efficiently targeted for degradation using LYTAC technology^[46]. More recently, a new class of LYTAC was designed to enable protein degradation via asialoglycoprotein receptor (ASGPR). Such protein is abundantly expressed only in hepatocytes, thus mediating increased LYTAC internalization in hepatocellular carcinoma cells (HCCs) compared to first-generation degraders.

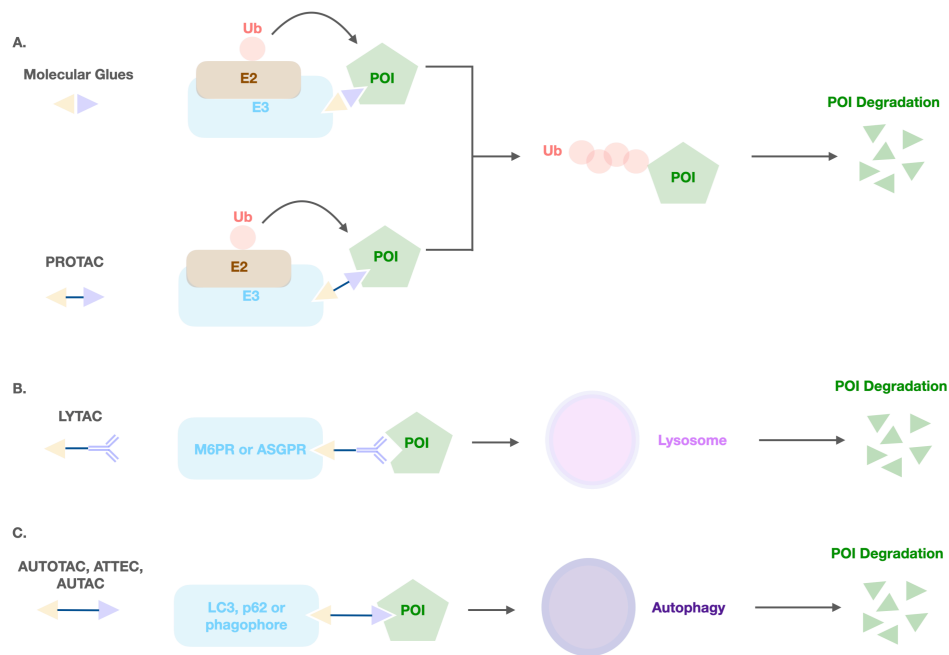


Figure 1.2: Targeted Protein Degradation Approaches - On panel **A** are reported strategies based on the UPS pathway. Both PROTAC and molecular glue technologies promote interactions between an E3 ubiquitin ligase and a POI, mediating target ubiquitination and elimination through proteasome. Panel **B** depicts LYTAC MoA. These bivalent molecules present on one side recruiter for transmembrane receptor CI-M6PR conjugated to an antibody or small molecule targeting a POI. Such technology enables targeted protein internalization via endocytosis and subsequent degradation in the lysosome. Panel **C** illustrates methods exploiting the autophagy-lysosome pathway. These approaches promote interactions between a POI and autophagy-related proteins (ATGs), inducing the degradation of the target protein through the autophagic process. Figure adapted from Zhang et al^[43].

This improvement allowed the authors to observe a complete target ablation at a concentration of just 10 nM. However, even higher doses were well-tolerated without any reported "hook effect" or liver toxicity in mice^{[47][48]}.

LYTAC technology remarkably enriched the protein degradation field by including extracellular proteins in the range of targets. In the future, LYTACs' sugar-based structure should be optimized while enlarging lysosomal receptors exploited to empower LYTAC technology further^[42].

Target ubiquitination serves as a code to prompt substrate degradation through proteasome or autophagic pathway. The latter also accounts for antimicrobial activity for group A streptococcus elimination, also known as xenophagy. Specifically, S-guanylation and ubiquitination trigger the recognition and degradation of bacterial proteins via the autophagy system^{[49][50]}. By mimicking this antibacterial mechanism, researchers attached a guanine-like structure to a POI ligand, giving rise to the first autophagy-targeting chimeras (AUTACs) (Figure 1.2). AUTACs effectively promoted the ubiquitination and degradation of proteins such as MetAP2 and FKBP12. AUTAC degradation ability was also tested on nuclear proteins, demonstrating the applicability of this technology on a wide range of substrates. Importantly, the autophagy lysosomal pathway is also responsible for organelle elimination, expanding the targeted degradation to bigger cellular components. In this context, the first mito-AUTAC efficiently eradicated damaged mitochondria, unlocking new therapeutic interventions for mitochondrial-related diseases^[51].

While AUTACs open new possibilities for exploiting degradation tools to remove organelles, their mechanism is more complex and results in slower elimination compared to PROTAC technology. Although AUTACs hold great potential for degrading larger cellular components, further refinement of their design and investigation of their mechanism of action is necessary for future applications^[52].

The array of degraders exploiting the autophagy pathway also includes autophagosome-tethering compounds (ATTECs). This class of molecules hijack LC3 protein to promote target internalization in the autophagosome and subsequent lysosomal degradation (Figure 1.2)^[53]. LC3 belongs to the broader family of autophagy-related (Atg) proteins, which control autophagosomal formation and expansion. In detail, LC3 is embedded in the autophagosomal membrane and fosters the connection between the cargo and the autophagosome^[54]. Similarly, ATTECs recruit target proteins to the autophagosome membrane through LC3 and promote its engulfment and degradation by the autophagolysosomal pathway. One of the most relevant studies reported the development of ATTECs for mutant Huntington protein (mHTT). Such compounds resulted active in the nanomolar range, promoting the elimination of mHTT exclusively without any wtHTT degradation observed. Furthermore, additional studies confirmed that developed degraders successfully crossed the blood-brain barrier (BBB), showing the potential of ATTEC technology for treating neurodegenerative pathologies like Huntington’s disease^[53]. Interestingly, this new tool extends beyond classical protein targets, achieving lipid droplet degradation. Such organelles are found in fat cells and tend to accumulate in various pathologies^[55].

Similarly to ATTECs, autophagy targeting chimera (AUTOTAC) technology enables POI degradation by prompting the autophagolysosomal system. Unlike ATTEC, this tool recruits p62 protein, playing an upstream role in the LC3-mediated macroautophagy (Figure 1.2)^[56]. In particular, p62 self-oligomerizes and activates upon target recognition. Consequently, oligomers interact with LC3 on the autophagosomal membrane, mediating cargo engulfment and elimination^[57]. Researchers design AUTOTAC molecules capable of mediating ER β , AR, and MetAP2 elimination at low concentrations. In addition, degraders presented increased cytotoxicity and antimigrating ability compared to related inhibitors. Notably, AUTOTACs showed outstanding results in degrading Tau toxic aggregates. Mutated Tau was successfully eradicated upon 10 nM AUTOTAC treatment after 30 minutes, reaching the maximum degradation level within 3 hours. These results represent a significant advancement in the protein degradation field, filling the remaining gap in eliminating toxic protein aggregate^[56].

An alternative tool to PROTAC technology is Chaperone-mediated protein degrader (CHAMP). CHAMPs are bivalent ligands interacting with a target protein on one side and recruiting a chaperone complex on the other. Essentially, this tool undirectly engages E3 ligases by interacting with chaperone protein associated with a specific E3 protein. Similarly to PROTACs, CHAMPs facilitate the contact between a POI and an E3, promoting target degradation via UPS. CHAMPs recruiting heat shock protein 90 (HSP90) were designed to target BRD4. Developed BRD4-CHAMPs efficiently eliminate the POI and exert an antiproliferative effect on cancer cell lines. Notably, such compounds did not display any degradative effect on HSP90 and reduced the level of Myc, regulated by BRD4, up to 90% in xenograft mice models. A single administration of BRD4-CHAMPs successfully induced target degradation for up to seven days, demonstrating the potential of this approach in cancer treatment^[58].

Protein folding regulation is essential to prevent the formation of toxic unfolded aggregates and consequently maintain cellular homeostasis^[59]. Drawing inspiration from this basic

mechanism, Crews conceived the hydrophobic tag (HyT) approach. This system holds a small hydrophobic moiety attached to a ligand for a POI. Therefore, when HyTs interact with a target protein, the small hydrophobic component destabilises the protein structure and mediates target degradation^{[61][62][63][64]}. Most commonly used hydrophobic tags contain adamantane, fluorene, pyrene, or Boc3Arg structures, and they successfully enabled ablation of HER3, AR, EZH2, and PAPR1 proteins^[61]. In a more recent study, researchers conducted a screening to find the best ALK degrader, ending up with a norbornene hydrophobic tag. In this work, developed HyT displayed an improved pharmacokinetic profile compared to ALK PROTAC^[65]. Unlike PROTACs, hydrophobic tags hold favourable pharmacokinetic properties and a reduced molecular weight. However, their substantial hydrophobicity limits HyT's capability to permeate cell membranes. In conclusion, while HyTs represent a powerful tool for targeted protein degradation, future efforts are needed to enhance their chemical and physical properties for broader therapeutic applications^[66].

Over the years, the targeted protein degradation field has welcomed a variety of new approaches, further improving degraders' potential. Among these technologies, antibody-based PROTACs (AbTACs) stood out for their ability to include cell-surface proteins in the range of targets. AbTACs operate similarly to PROTACs by simultaneously recruiting an E3 and a POI. Nevertheless, bispecific IgGs replace classical small molecule ligands, enabling cell-surface protein elimination by membrane-bound E3 ligase engagement. First reported AbTAC successfully promoted the degradation of PD-L1. Developed AbTAC facilitated target protein lysosomal elimination by fostering the interactions between RNF43 ligase and PD-L1^[67]. AbTAC technology just landed in the protein degradation field, and further advancements are expected in the future.

Additional tools controlling protein degradation comprise bispecific aptamer chimera (BIAC) and GlueTAC. Both rely on lysosomal elimination and target membrane proteins. In the case of BIAC technology, degradation is promoted by a three-component molecule holding two aptamer regions bridged by a DNA-based linker. Such compounds mediated mesenchymal-epithelial transformation (Met) receptor and tyrosine kinase-like protein 7 (PTK-7) elimination through lysosome-shuttling receptor (IGFII R) engagement. Aptamers hold the advantage of valid and easy synthesis while retaining correct stability^[68]. GlueTAC, on the other hand, recruits target proteins using an amino acid-based nanobody and directs them for lysosomal degradation via a lysosomal sorting sequence (LSS). Thus, GlueTAC presents a reduced molecular weight compared to similar approaches thanks to the nanobody-based structure^[69]. Although BIAC and GlueTAC tools ensured membrane protein degradation, further studies are needed to define their safety profiles and optimize their therapeutic potential^[42].

The latest technology involving ternary complex formation is known under the name of Regulated induced proximity targeting chimeras (RIPTACs) and holds great promise in cancer treatment. Although this approach does not lead to POI degradation, similar to PROTACs, it induces protein-protein interactions (PPIs) and promotes cancer cell death. In detail, RIPTACs interact with a protein fundamental for cell survival (EP), while engaging a specific protein expressed exclusively in cancer cells (TP). Thus, EP proteins are held together in complex with TP ones through RIPTAC molecules, causing an impairment in EP function and ultimately leading to cellular death. Crews group validated RIPTAC technology by a sophisticated engineered system, demonstrating their ability to trigger death uniquely in cancer cells^[70]. In conclusion, this groundbreaking approach has inspired the creation of HALDA Therapeutics, a company focused on leveraging RIPTACs for precision oncology^[71].

Table 1.1: Summary of the different TPD technologies, reporting features and defects associated with each technology. Adapted from Liang et al^[6]

Technology	Degradation System	Features	Defects
CHAMP	UPS	Precision, Specificity	Narrow application range, early development
HyT	UPS	Small molecule weight, higher security	Poor physical and chemical properties, low degradation efficiency
AbTAC		Targeting membrane proteins, high biospecificity	High costs, potential immunogenicity, unclear endocytosis mechanism
GlueTAC		Small overall size, high efficiency, wide applicability	Safety and half-life need to be investigated
AUTOTAC	Autophagy-lysosomal pathway	Degrade misfolded proteins, degradable aggregate proteins	In the initial stage
AUTAC	Autophagy-lysosomal pathway	Direct tethering allows precise degradation without tagging, suitable for challenging targets	Less specific and harder to develop, potential off-targets related to tethering mechanism
ATTEC	Autophagy-lysosomal pathway	Effective on broader range of targets, including organelles	Potentially less selective and slower compared to PROTACs
Molecular GLUEs	UPS	Potentially good oral bioavailability, improved cell permeability and metabolic stability	Serendipity-based discovery

1.1.3 The Chemistry Behind PROTAC Synthesis

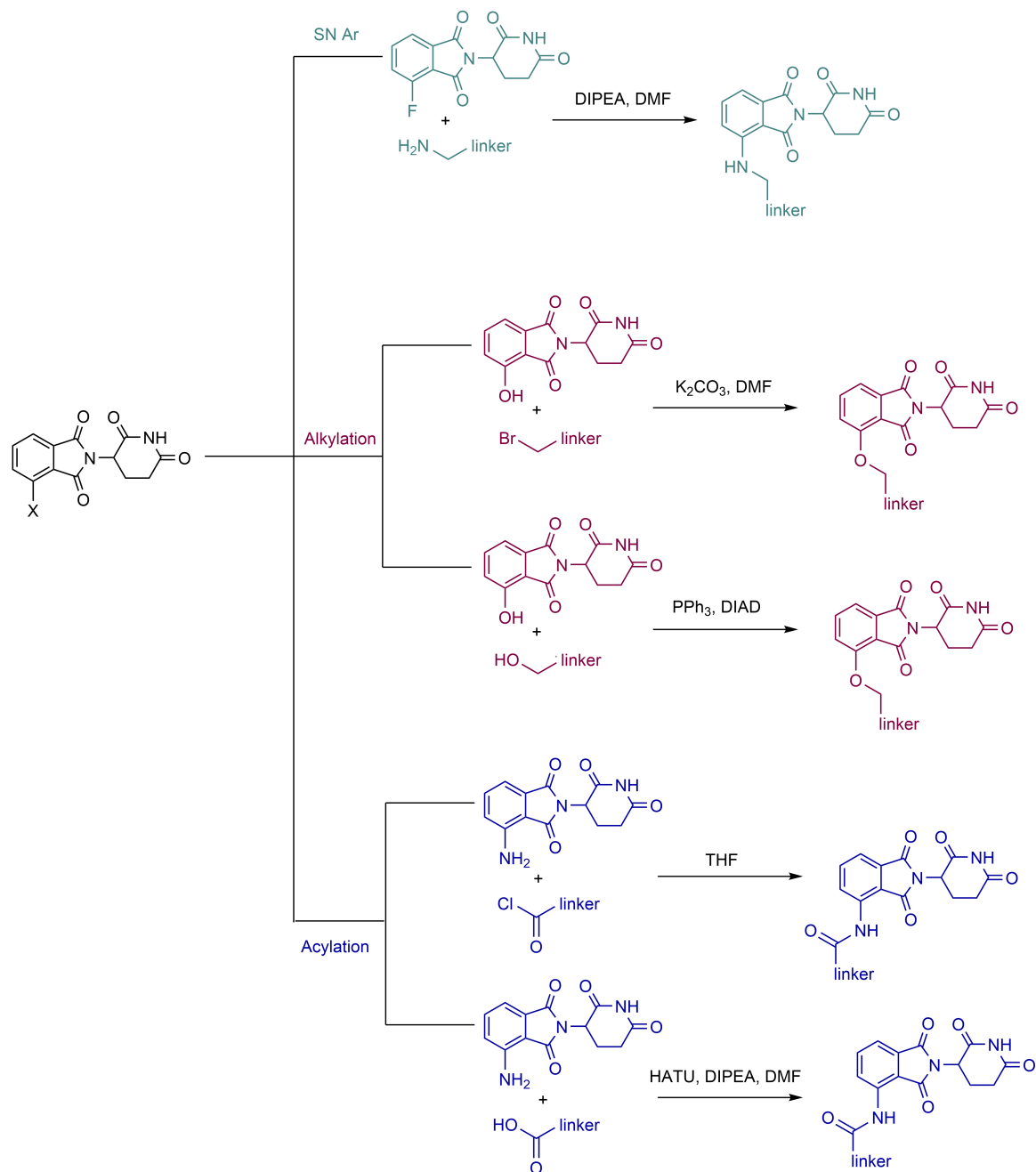
Different approaches are employed in PROTAC synthesis, depending on the specific components and design of the degraders.

The linker attachment to the E3 recruiting moiety occurs via amine, amide or ether bond formation in CRBN-based degraders. Since the repurposing of thalidomide for its ability to recruit CRBN ligases, such scaffold is usually employed in PROTAC development. The main strategies in the attachment of the linker are nucleophilic aromatic substitution, alkylation, and acylation (Scheme 1.3). The former involves the exploitation of fluorinated thalidomide derivatives and linkers holding primary amine as a terminal group. Reactions are performed using polar aprotic solvents like dimethylsulfoxide or dimethylformamide (DMSO, DMF) in the presence of N,N-diisopropylethylamine (DIPEA) to neutralize hydrofluoric acid forming during the reaction^[72]. The alkylation strategy is suitable for 4-hydroxythalidomide derivative, holding a phenolic group reacting with alkyl halide linkers^[73]. Alternatively, the phenolic group could react with primary or secondary alcohols catalyzed by triphenylphosphine (PPh₃) and diisopropyl azodicarboxylate (DIAD), according to the Mitsunobu reaction^[74]. Linker attachment through amide bond formation concerns the employment of pomalidomide or lenalidomide derivatives, amenable for acylation reactions. The reaction can be performed using acyl chloride linkers or directly carboxylic acids in the presence of coupling reagents (3-oxid hexafluorophosphate or propanephosphonic acid anhydride)^{[75][76]}.

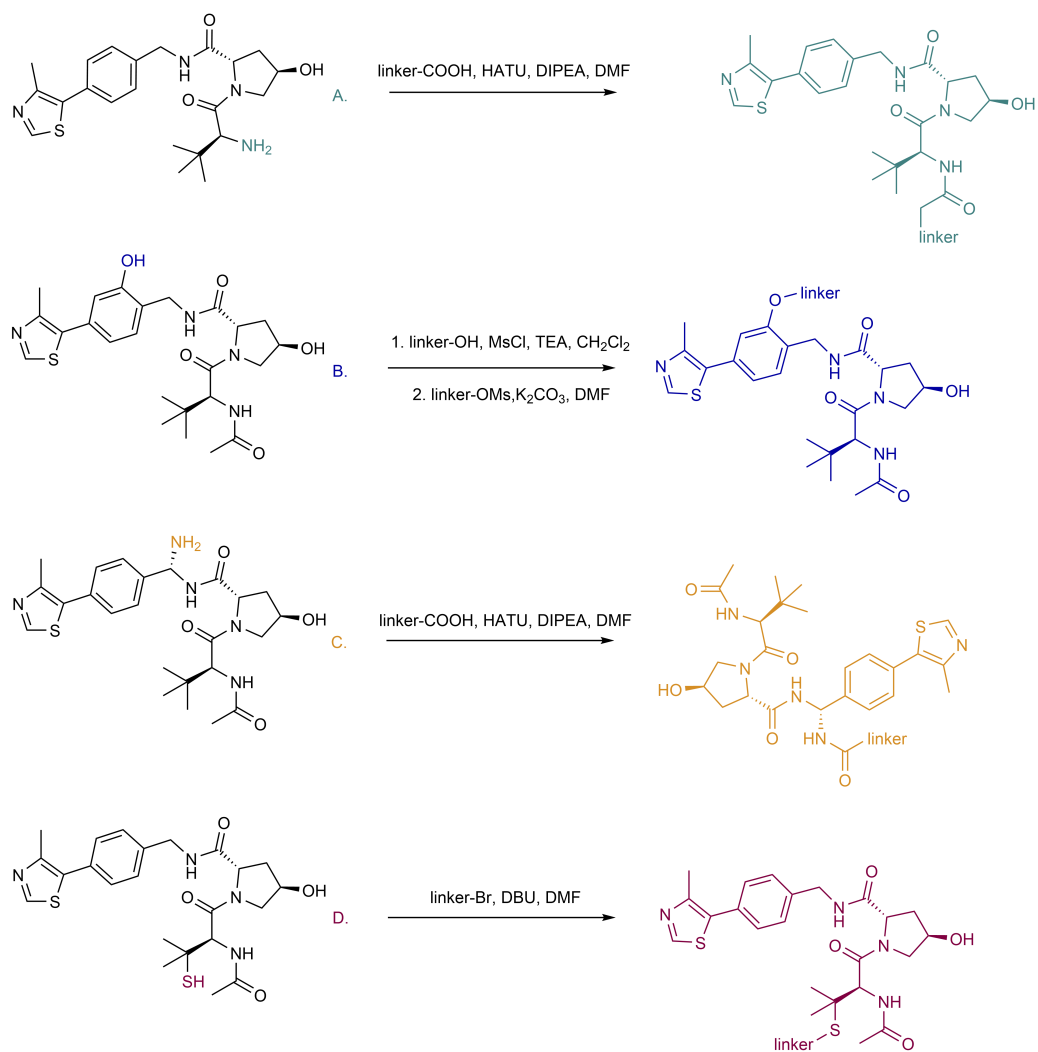
Another widely exploited ligase in the development of PROTACs is VHL. Currently used recruiters encompass VH032 scaffold and derivatives, holding four anchoring points to host linker structure. The most common attachment occurs through an amide bond formation between a linker with carboxylic acid as a terminal group and the amine in position 2 of VH032. Similarly to CRBN-based PROTACs, acylation is performed via coupling reaction (Scheme 1.4, A.)^[77]. Another strategy involves linker attachment through the phenolic linkage point via ether bond formation, mediated by a mesylate precursor (Scheme 1.4, B.)^[78]. The third path exploits linker attachment via the benzylic methylene group through a coupling reaction similar to the first described strategy (Scheme 1.4, C.)^[79]. The last anchoring point involves thioether bond formation between a thiol substituent in the E3 ligase recruiter and an alkyl halide linker (Scheme 1.4, D.)^[80].

Regarding linker structure, the great majority of PROTACs hold alkyl or PEG-like linkers of various lengths, allowing a high level of rotational freedom of the other two PROTAC components (POI ligand and E3 recruiting moiety). Conversely, fewer heterobifunctional degraders present a rigid type of linker. Scientists introduce rigidity through aromatic, piperidine, piperazine, or triazole rings. Notably, the latter is obtained by exploiting a "click" chemistry reaction, specifically azide-alkyne cycloaddition^[81].

The binding to the POI ligand is largely determined by the functional groups present within its structure. In most cases, the formation of amine or amide bonds is reported, utilizing the same synthetic methodologies typically employed for the attachment of E3 recruiting moiety to linkers^{[82][83]}.



Scheme 1.3: Common synthetic strategies in linker attachment for CRBN-based PROTACs.



Scheme 1.4: Common synthetic strategies in linker attachment for VHL-based PROTACs.

1.2 BAZ Bromodomains

1.2.1 Bromodomains: Structural and Functional Features

Bromodomains (BrDs) discovery dates back to 1992 when the Tamkun group identified the Brahma gene in *Drosophila* models^[84]. Only later, this module was found recurrently in chromatin and transcription-linked proteins, suggesting a possible role of BrDs in transcriptional regulation^[85]. Nevertheless, the final demonstration occurred in 1999 when the first PCAF (p300/CBP-associated factor) bromodomain structure was determined through NMR spectroscopy. BrD structure revealed a unique configuration of four left-handed helix bundles, differentiating these proteins from the more common right-handed four-helix bundle fold. In detail, the four helices (α Z, α A, α B and α C) interconnect through a couple of loops named ZA and BC, which form a hydrophobic core and possibly contribute to the left-handed four-helix bundle stability (Figure 1.5)^[86]. In the same year, researchers proved bromodomains' binding ability to acetylated lysine residues (Kac)^[87].

Additional insights on bromodomain structure came from an extensive study in 2012, showing BrD preferential binding to various acetylated lysines in core histones. In particular, ZA and BC loops present the highest sequence variability among the different BrDs, indicating their role in directing recognition to specific Kac. However, Kac binding mode is conserved between the various BrDs, involving the formation of two fundamental hydrogen bonds. Notably, the carbonyl oxygen of Kac establishes interactions directly with the amine group of the asparagine (Asn) side chain and indirectly via water with the hydroxyl group of the tyrosine (Tyr) in BrD (Figure 1.6). This modality occurs repeatedly in most bromodomains, with only a few reported exceptions presenting Tyr, threonine (Thr) or aspartic acid (Asp) instead of standard Asn residue. In addition, Kac recognition takes place also through a set of water molecules found at the bottom of the Kac pocket. Such molecules mediate hydrogen bond formation between Kac and BrD; alternatively, they form a solvation shell protecting hydrophobic and aromatic residues in the Kac unbound state^{[88][89]}.

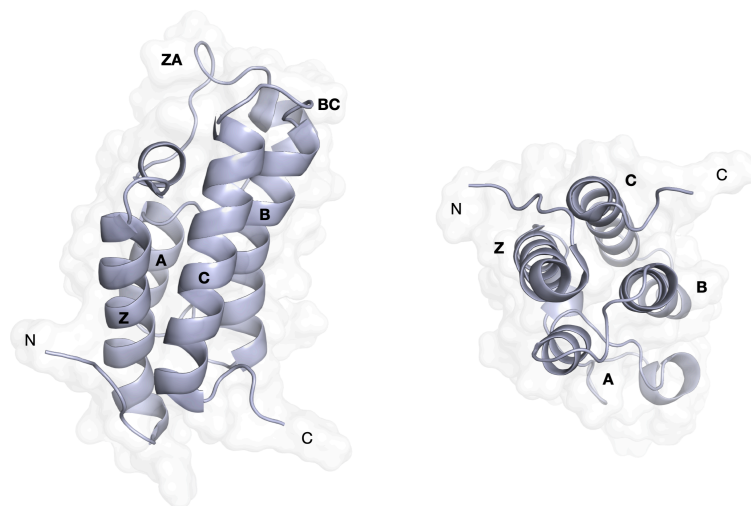


Figure 1.5: Bromodomains Structure - Ribbon diagram of PCAF BrD (PDB 1N72) NMR spectroscopy structure. The left-handed four-helix bundle is depicted in light green, and different helices are reported on the structural representation. The inter-helical ZA and BC loops are labelled in the illustration. Figure adapted from Zaware et al^[89].

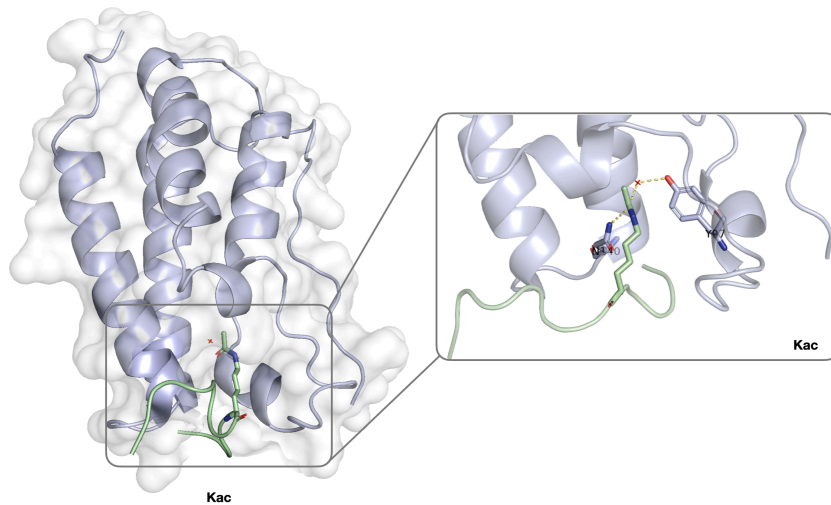


Figure 1.6: Kac Recognition - Ribbon representation of BRD4-BD1 in complex with Kac (PDB 3UVW) crystal structure. Kac and side chains involved in the recognition are labelled in the structural representation. Figure adapted from Zaware et al^[89].

The human genome accounts for 61 BrD genes, giving rise to 46 distinct proteins^[88]. With time, bromodomains were divided into eight main subfamilies according to their sequence. As chromatin "readers", BrDs exert pivotal functions in transcription initiation or elongation, acting at the level of chromatin remodeling, histone modifications, and recruitment of transcription factors (Table 1.2)^[90]. Most BrDs belong to larger complexes and are often associated with other conserved modules, working as an independent unit in transcription regulation. The more relevant families include group IIIb and group V. The former holds Imitation SWItch (ISWI) family chromatin remodeling complexes, while the latter involves Bromodomain and Extra-Terminal Domain (BET) members^[89].

In detail, Group IIIb components are BAZ1A (ACF1), BAZ1B, BAZ2A, BAZ2B, BPTF and CECR2 BrDs^[91]. ISWI complex functioning depends on ATPase activity exerted by either SNF2H or SNF2L. According to the core ATPase, ISWI complexes recruit different accessory subunits. For instance, SNF2H assemble with nucleolar remodeling complex (NoRC), WSTF ISWI chromatin remodeling (WICH), ACF and human CHRAC complexes, while SNF2 is found in NURF and CECR2-containing remodeling factor (CEFR) complexes^[92]. In general, group IIIb members play roles in assembling or spacing nucleosomes^{[93][94]}.

On the other hand, group V comprises BRD2, BRD3, BRD4, and testis-specific BRDT BrDs, all belonging to the well-known BET family. In structural terms, BET present a couple of adjacent bromodomains followed by the ET domain. Consequently, this class of BrDs preferentially recruit proteins holding contiguous tandem Kac^[95]. BET BrDs function is not limited to Kac recognition but also involves transcription factor recruitment in concert with the ET domain. Normally, this group of BrDs promotes transcription by establishing open chromatin features. Due to its role in enhancing oncogenes or pro-inflammatory cytokines/chemokines transcription, BRD4 is the most studied member of the family^{[96][97][98]}.

By controlling important equilibria in gene transcription, the BrD family gained more attention and emerged as new potential targets in different pathologies treatment^[89].

Table 1.2: Different BrDs group members and their related functions. Table adapted from Zaware et al^[89]

Group	Members	General Function
I	PCAF, GCN5L2, p300/CBP, TAF1, TAF1L, BRPF1A/B, BRPF2, BRPF3, BRD8	Histone lysine acetylation
II	ASH1L, MLL	Histone lysine methylation
III	SMARCA2A/2B, SMARCA4, PRBM1, BRD7, BRD9, BAZ1A, BAZ1B, BAZ2A, BAZ2B, BPTF, CECR2	Chromatin remodeling
IV	ATAD2, ATAD2B	Replication-coupled chromatin assembly
V	BRD2, BRD3, BRD4, BRDT	Transcriptional activation
VI	TRIM24, TRIM28, TRIM33A/B, TRIM66	Protein sumoylation, ubiquitination, degradation
VII	SP100, SP110A/C, SP140, SP140L	PML nuclear bodies, transcriptional regulation
VIII	ZMYND8, ZMYND11	Transcriptional repression, DNA repair
IX	BRWD1, BRWD3, PHIP	Cell shape control, insulin regulation

1.2.2 Targeting Bromodomains: Current Approaches and Latest Advances

As anticipated, BrDs control essential mechanisms spanning from chromatin remodelling to transcription regulation; not surprisingly, BrD-containing proteins (BCPs) contribute to pathological conditions in their altered state^[99]. Reported disorders associated with BCP abnormalities include cancers, inflammation, cardiovascular diseases, and viral infections ^{[89][100][101][102]}. For instance, studies demonstrated that BRD2, BRD4, ATAD2, or KAT2A overexpression correlates with tumour progression and aggressiveness, thus including these proteins in the oncogenes list^{[103][104][105]}. All this evidence suggested that BCPs modulation could serve as a new approach to treating multiple disorders, leading to the release of numerous molecules targeting BCPs. The most employed strategy blocks BCPs' pathological function by interfering with Kac recognition exerted by the BrD domain, mimicking Kac interactions in the active pocket. The array of BCPs inhibitors divides into two major subgroups: non-BET inhibitors and BET ones. The latter could split further into pan-selective BET inhibitors, BRD4-selective inhibitors, and BD1/BD2-selective inhibitors (Figure 1.7)^[99].

In 1996, Mitsubishi Tanabe Pharma Corporation of Japan successfully developed a series of thienotriazolodiazepine-based compounds for treating inflammatory bowel diseases^[106]. Later, additional studies showed that thienotriazolodiazepines promoted cancer cell death in mammals, ascribing this effect to Kac recognition impairment^[107]. Based on these results, researchers exploited the thienotriazolodiazepine scaffold to design the first BET inhibitor. The above, known as (+)-JQ1, presented a thieno-triazolo-1,4-diazepine structure and displayed an affinity in the nanomolar range for BET proteins. Structural characterization elucidated the JQ1 binding mode, highlighting H-bond formation between the JQ1 triazole ring and Asn (Asn140 in BD1, Asn429 in BD2), similar to Kac recognition (Figure 1.8). In addition, this inhibitor established hydrophobic interactions with ZA and BC loops, stabilizing the complex. On the contrary, (-)-JQ1 presented

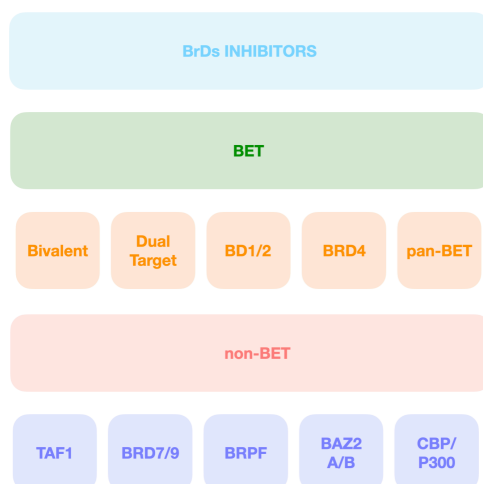


Figure 1.7: Inhibitors Targeting Bromodomains - A schematic representation of BCPs inhibitors divided into BET and non-BET subclasses. For each category, the main strategies or targets exploited in BCP targeting are reported. Figure adapted from Pan et al^[114].

unfavourable structural hindrances, clashing with Leucine 92 (Leu) and Leu94 residues in the Kac pocket^[108]. JQ1 treatment enhanced the survival rate in NUT midline carcinoma, rare aggressive squamous cell epithelial cancer characterized by NUT gene fusion with BRD4 or BRD3. At the same time, JQ1 treatment slowed cancer cell proliferation by downregulating MYC expression in multiple myeloma^{[109][110][111]}. Unfortunately, such compounds present reduced half-life, resulting in limited use as research tools. However, the thienotriazolodiazepine group of molecules enlarged, leading to the development of fluorinated alternatives^[112]. The latter presented improved metabolic stability while gaining BBB permeability, a relevant property for BET-mediated central nervous system (CNS) disorders^[113].

Dimethyl isoxazole discovery casually occurred in 2011, similarly to pan-BET thienotriazolodiazepine-based inhibitors. Scientists found that N-methyl pyrrolidinone displayed weak BRD2/4 inhibition^[115]. Based on these results, researchers designed a series of new compounds containing N-methyl pyrrolidinone skeleton, culminating with approximately 22 developed molecules^[114]. The most potent, CF53, exhibited an antiproliferative effect on triple-negative breast carcinoma while presenting adequate oral bioavailability^[116].

Another group of pan-BET targeting molecules belongs to the class of tetrahydroquinoline. In general, this scaffold was already known for its ability to interact with Apo1 protein and induce target up-regulation. However, a screening campaign indicated tetrahydroquinoline derivatives like potent BET inhibitors^[117]. By performing a series of structural optimizations, scientists ended up with compound I-BET726, presenting a nanomolar affinity for BET proteins. In addition, structural characterization showed that I-BET726 recapitulated the Kac binding mode, establishing an additional H-bond with Lys107^[118]. However, the most advanced project involves compound CC-90010, currently tested in clinical phase I for solid tumours or refractory/relapsed non-Hodgkin lymphoma treatment^[119].

Even though pan-selective BET inhibitors demonstrated their validity as potent therapeutic agents, BRD4 resulted as the most attractive member of the family. Therefore, researchers devoted considerable effort to finding selective BRD4 ligands^[114], leading to the development of compounds like ZL0420 or FL-411. Such molecules exhibited strong preferential binding to BRD4 (30 to 120 fold selectivity over BRD2/3/T) while displaying antitumour activity^{[120][121]}.

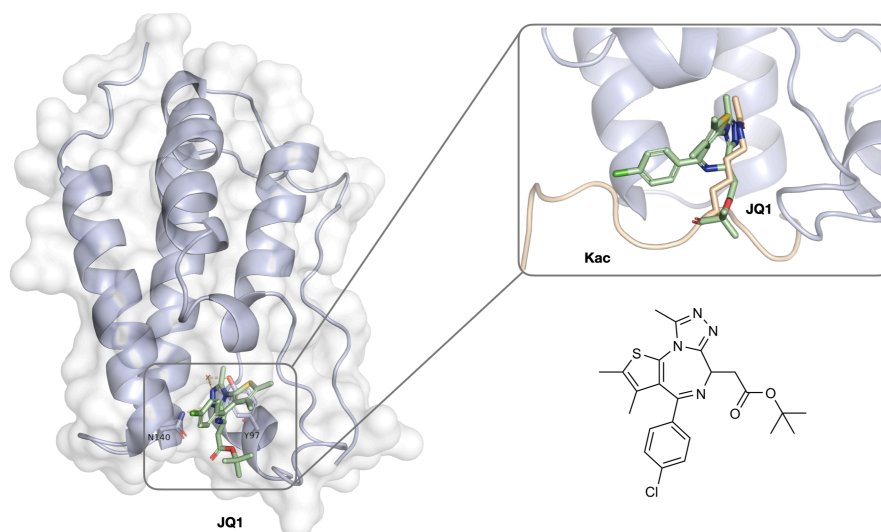


Figure 1.8: JQ1 Inhibitor - Figure reports a ribbon representation of BRD4 bromodomain in complex with (+)-JQ1 inhibitor (PDB 3MXF). The H-bond between Asn 140 and the triazole ring is depicted in pale yellow. Alongside is illustrated JQ1 and Kac (PDB 3UVW) superimposition, showing the similar binding mode displayed by the two ligands.

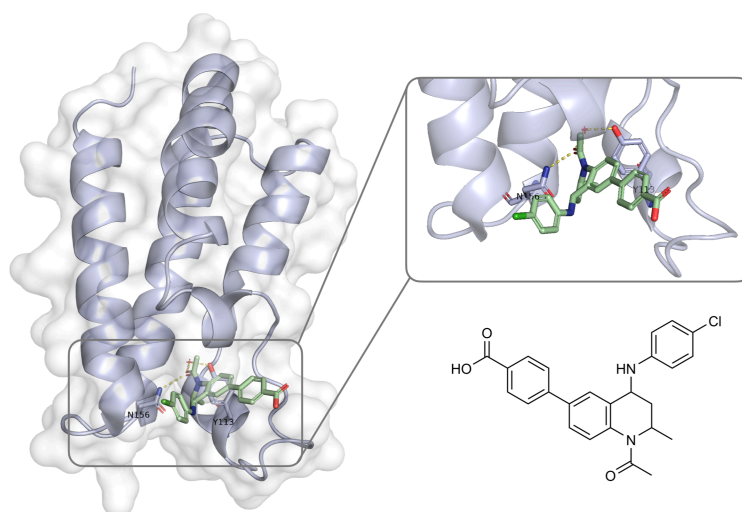


Figure 1.9: I-BET726 Inhibitor - Ribbon diagram of BRD2(BD1) bromodomains bound to I-BET726, residues involved in the interactions are labelled (PDB 4UYF).

An additional level of selectivity was still missing to elucidate the mechanism of action behind BET inhibition. As anticipated in chapter 1.2.1, the BET family features a couple of bromodomains localized in adjacent regions (BD1 and BD2). In particular, studies suggested a differential role associated with BD1 or BD2, revealing an anticancer effect related to BD1 inhibition while displaying anti-inflammatory activity upon BD2 function impairment^[122]. The

logical activity, their unfavourable physicochemical properties and reduced metabolic stability prevented their advancement^[114].

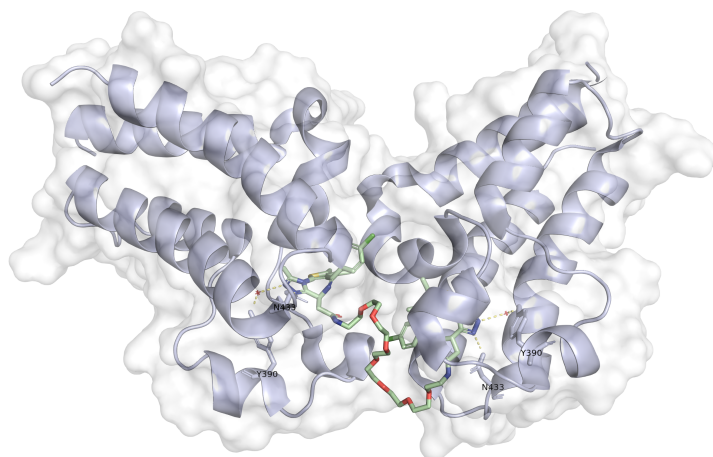


Figure 1.11: JQ1 Bivalent Inhibitor - Ribbon diagram of BRD4 bromodomains in complex with bivalent JQ1 molecule depicted in yellow (PDB 5JWM).

Among the different therapeutic opportunities involving BET inhibition, dual-target inhibition emerged as an efficient strategy to block cancer progression while counteracting drug resistance and reducing standard dosage. Several studies showed that BET ligands could elicit a beneficial outcome when matched with other protein inhibitors. Thus, simultaneous inhibition of two separate targets often produces a synergistic effect^[129]. Among the different targets concurrently inhibited with BET, scientists proposed proteins like histone deacetylases (HDACs), polo-like Kinase 1 (PLK1), phosphatidylinositol 3-kinase (PI3K), poly(ADP-ribose) polymerase 1 (PARP1), and CDKs^[114]. The former strategy allowed a simultaneous downregulation of c-MYC while raising H4 acetylation, stopping cancer cell growth in NMC and AML cell lines^[130]. Conversely, BI2536 dual BRD4 and PLK1 inhibition gave rise to an analogue effect in haematological malignant cells. Notably, BI2536 derivatives are currently under evaluation in clinical phase I and II for solid tumours and haematological malignancies^{[130][131]}. In synthesis, dual-target inhibition opened a new approach to cancer treatment, ensuring a reduced risk of developing resistance with milder dosages^[114].

The successful development of BET inhibitors stimulated the scientific community to explore the rest of BCPs. The reasons behind this investigation are related to elucidating BCP's biological functions and finding new possible targets in drug discovery^[133].

Among the different non-BET proteins, CBP/p300 gained increased attention when found dysregulated in cancer. In 2014, the Structural Genomic Consortium (SGC) discovered a selective CBP inhibitor^{[134][135]}, which led to CCS1477 development, currently tested in clinical phase I/IIa for solid tumours and haematological malignancies^[136].

Researchers spent considerable effort developing new inhibitors for PCAF/GCN5 BCP due to its relevant role in cancer, AIDS and inflammation^[137]. In particular, L-Moses resulted in active and selective PCAF inhibition, holding good metabolic stability and cell permeability. Additionally, the structural characterization of GCN5 in complex with L-Moses illustrated a series of interactions occurring in the Kac binding pocket, serving as a starting point for further structural modifications (Figure 1.12)^[138].

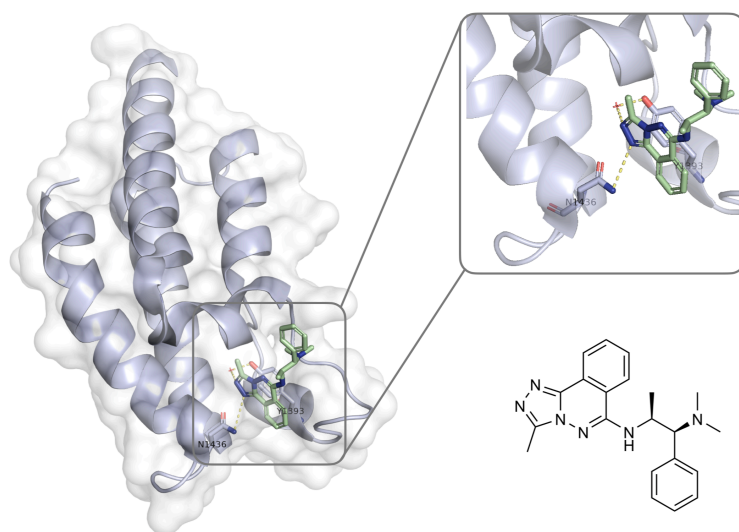


Figure 1.12: L-Moses - Ribbon representation of *pfGCN5* (Brd from *Plasmodium falciparum*, sharing 64% sequence with PCAF) bound to L-Moses depicted in light pink (PDB 5TPX).

BRD9 and BRD7 selective inhibition resulted impaired by their almost identical bromodomains until GSK developed the first BRD9 inhibitor. This molecule presented 200-fold increased selectivity for BRD9 compared to BRD7^[139]. Stimulated by this achievement, Genentech and Constellation Pharmaceuticals conceived another BRD9 selective inhibitor; however, this did not display the expected biological activity^[140]. Conversely, dual BRD7/9 inhibition, exerted by the LP99 compound, showed anti-inflammatory potential in human monocytic leukaemia cells^[141]. Similarly, TP-472 decreased melanoma cell growth *in vitro* and *in vivo* by inhibiting BRD7 and BRD9^[142].

BRPF members are multi-domain proteins belonging to large complexes, challenging scientists to define their cellular role. Thus, BRPF inhibition represented a pivotal strategy to explain and clarify their physiological functions. Notably, GSK developed a selective BRPF1 compound composed of a benzimidazole scaffold^[143]. Meanwhile, SGC conducted a screening campaign that allowed the discovery of three pan-BRPF inhibitors^[144]. In addition, developing new therapeutic agents targeting BRPF became urgent when these proteins were related to cancer, inflammation and osteoclastogenesis^[145].

In conclusion, BCPs emerged as relevant targets in many pathologies, opening new strategies in cancer and anti-inflammatory fields. Exploited approaches mainly rely on Kac pocket occupation to impair BCPs' disease-related functions. In addition, the development of inhibitors highlighted residues involved in the selective binding to BrDs, guiding future structural modifications. Unfortunately, none of the reported compounds is currently on the market, but novel technologies like PROTACs or strategies as bivalent ligands are promising alternatives to speed up the commercialization process^[114].

1.2.3 Targeted Degradation of Epigenetic Readers: A Novel Pharmacological Strategy

Epigenetic modulators indirectly control gene expression through a complex interplay between "writers," "erasers," and "readers." Such an intricate landscape requires a tight level of regulation, which inevitably leads to disease pathogenesis upon alteration^[146].

The best strategy to investigate epigenetic modular involvement in pathological conditions takes advantage of genome-wide knockdowns (RNA interference or CRISPR) while shedding light on possible therapeutic interventions. In particular, knockdown screening campaigns highlighted the pivotal role of epigenetic regulators in different pathologies and suggested their inhibition as a valid therapeutic strategy^{[147][148]}. Although this approach worked appropriately with some targets, scientists discovered that simple inhibition did not recapitulate knockdown experiments with some epigenetic modulators. To explain the lack of effect upon inhibition researchers hypothesized that the engagement of a single domain belonging to multi-subunit proteins, like epigenetic modulators, does not affect the entire functioning of the target. Thus, strategies involving overall complex elimination could overcome inefficient inhibition. Therefore, researchers exploited PROTAC technology to degrade epigenetic modulators, inducing the interactions between an E3 ligase and the most druggable/susceptible subunit. In addition, PROTAC technology ensured more selective targeting compared to inhibitors, a relevant feature in epigenetic modulators that display a high level of sequence homology^{[146][149]}.

Due to their pivotal role in different pathologies, BCPs were among the first epigenetic modulators successfully eliminated by PROTAC technology. As reported in Chapter 1.2.2, the BET family accounts for the highest number of developed inhibitors, enticing scientists to design a related PROTAC degrader. The first degrader targeting BRD4 (dBET1) held a JQ1 BET inhibitor and a thalidomide-based CRBN ligand, conjugated through a N-butyl-2- hydroxyacetamide linker. dBET1 prompted BRD4 degradation with a DC_{50} of 0.43 μ M, impairing cancer progression in leukaemia mice models^[150]. Notably, the first degrader served as a starting point for designing different compounds holding increased target engagement ability or cellular permeability. In addition, the first ternary complex structure determined by the Fisher group guided further structural modifications. In particular, scientists achieved an increased BRD4 selectivity by reducing linker length and inducing structural clashes with the other off-targets (Figure 1.13)^[151]. With time, the array of exploited E3 ligases for BRD4 degradation enlarged, including VHL^[80], RNF114 (through covalent interaction)^[152] and MDM2. PROTAC-mediated degradation of the latter displayed a substantial anti-proliferative effect through BRD4 elimination while stabilizing tumour suppressor p53, an MDM2 interactor^[153]. Among the different PROTACs targeting BRD4, alternative degrading strategies emerged recently. In this context, scientists conceived the first antibody conjugate BRD4 degrader^[154]; meanwhile, Lu and collaborators designed a BRD4 ATTEC, promoting target degradation through lysosomal pathways^[155]. The latest improvements include the BRD2 trivalent PROTACs and click-formed proteolysis targeting chimeras (CLIPTACs). The former achieved BRD2 degradation by simultaneous engagement of BD1 and BD2 bromodomains^[156]. The latter solved the PROTAC MW problem by administering the two recruiting moieties separately and promoting bivalent compound formation through click-reaction inside cells^[157].

Another BrD family component efficiently targeted by PROTAC technology is BRD9. This protein is part of the Switch/ Sucrose-Nonfermentable (mSWI/SNF) chromatin remodeling complex, recently associated with tumoural and neurological disorders^[102]. In the case of BRD9, CRISPR screening revealed a susceptibility of this BrD in pediatric malignant rhabdoid tumours^[158]. Scientists obtained the first BRD9 PROTAC by an iterative approach employing a previously identified ligand (GSK-39). Later, the combination of co-crystal PROTAC-BRD9 structures and

in-silico ternary complexes modelling allowed the discovery of a second BRD9 degrader. Surprisingly, PROTACs encompassing GSK-39 in their structure mediated BRD9 degradation only by recruiting CRBN^[159]. In a subsequent article, researchers designed a dual BRD9/BRD7 PROTAC by exploiting a promiscuous ligand. Differently from GSK-39-based PROTACs, the developed degrader engaged VHL ligase to hijack target degradation. The improved degradation profile observed with VHL-based degraders should be ascribed to an increased cell permeability^[160].

As was the case for BRD9, genome-wide knockdown screening identified SMARCA2 and SMARCA4 as essential components of the BAF remodeling complex, whose targeted degradation induces cancer cell death^[161]. These results prompted scientists to design the first SMARCA2/4 PROTAC degrader, ACBI1. The latter held VHL recruiting moiety conjugated to SMARCA2/4 known ligand. By efficiently eliminating BAF remodeling complexes, ACBI1 displayed a potent anti-proliferative effect in different cancer cell lines with target affinity in the nanomolar range (IC_{50} 28 nM) (Figure 1.14)^[162].

Additional chromatin readers targeted by PROTACs outside the BET family comprise CBP and p300. These two proteins act as histone acetyltransferases (HATs), containing a BrD region acting as a Kac reader and sharing 96% sequence similarity. The involvement of CPB/p300 in different cancer features, like cell proliferation or metastatic potential, suggested their potential role as new targets^[162]. Despite the development of potent CPB/p300 ligands, exclusive BrD inhibition failed to produce an anti-proliferative effect. Therefore, researchers adapted the CPB/p300 inhibitor into a PROTAC degrader. Currently, the JET-209 degrader efficiently promotes CPB/p300 degradation, leading to tumor growth suppression in xenograft models^[163].

In conclusion, degrading BCPs represents a powerful strategy to disrupt their pathology-related functions. PROTAC technology enables targeted BCP elimination by engaging simultaneously an E3 ligase and the bromodomain, making BCPs a suitable target for this innovative approach^{[146][149]}.

Current PROTAC-based strategies targeting epigenetic readers are summarized in Table 1.3.

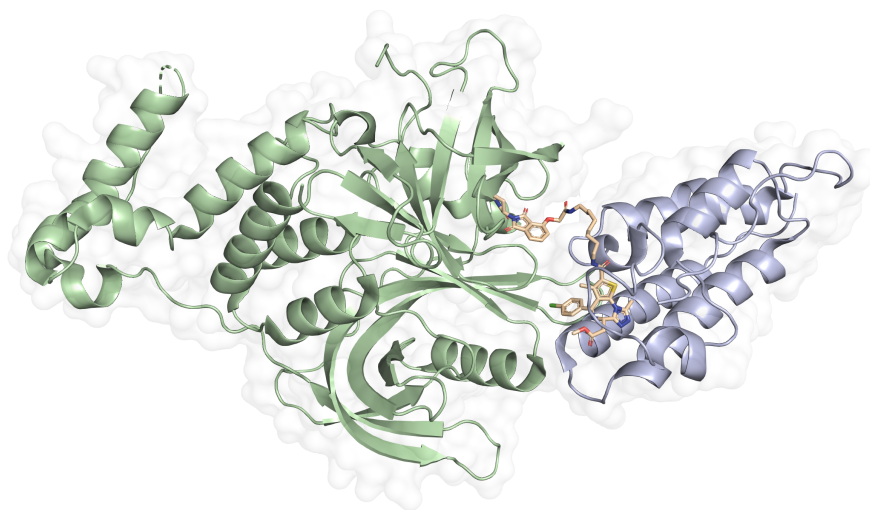


Figure 1.13: *dBET23* PROTAC - Ribbon representation of CRBN (light green) and BRD4 bromodomain (light blue) in complex with *dBET23* (orange) PROTAC (PDB 6BN7).

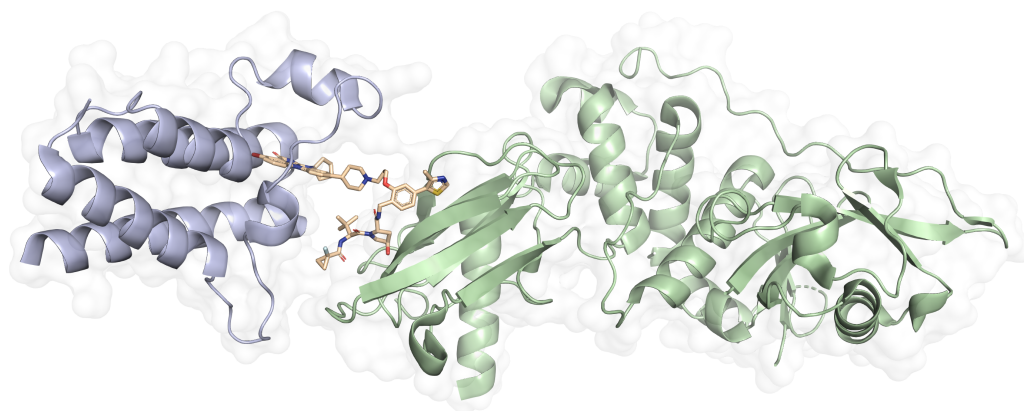


Figure 1.14: ACBI1 PROTAC - Ribbon representation of pVHL-EloB-EloC (light green) and SMARCA2 bromodomain (light blue) in complex with ACBI1 (orange) PROTAC (PDB 7Z6L).

Table 1.3: Developed degradation strategies involving different epigenetic "readers".

Epigenetic Targets	TPD Technologies
BRD4	PROTAC, Ab-PROTAC, CLIPTAC, ATTEC, pc-PROTAC, SNIPER, photoPROTAC
BRD2	photoPROTAC, trivalent PROTAC
BRD9	PROTAC
BRD2/3/4	PROTAC
CBP/p300	PROTAC
SMARCA2/4	PROTAC

1.2.4 BAZ2A Bromodomain Function as Part of NoRC Complex

BAZ2A (bromodomain adjacent to zinc finger domain protein 2A), or TIP5 (TTF-I interacting protein 5), is a 1908 amino acids protein featuring several domains devoted to DNA-protein interactions. The domain structure of BAZ2A comprises an MBD (methyl-CpG-binding domain), a series of AT-hook DNA-binding domains and DDT (DNA binding homeobox and different transcription factors) domain. In addition, the C-terminal region accommodates a PHD (plant homeodomain) zinc finger domain adjacent to a BrD, acting as an independent module in the Nucleolar Remodeling Complex (NoRC) functioning^[165] (Figure 1.15). The latter promotes the silencing of some rRNAs, mediating the establishment of repressed chromatin features. In particular, rRNA silencing relies on the Kac16 on histone 4 (H4K16ac) recognition exerted by BAZ2A BrD. Therefore, point mutation on BAZ2A BrD led to an impairment in NoRC function, suggesting the pivotal role of Kac recognition in activating the complex. Once acH4K16 identification occurs, the BrD and the PHD domain cooperate by recruiting factors like histone deacetylase1 (HDAC1) and DNA methyltransferase 1/3b (DNMT3b), establishing heterochromatin features. In addition, the BrD-PHD unit also interacts with SNF2h, another NoRC subunit working as ATPase and promoting chromatin remodelling (Figure 1.16)^{[166][167][168]}. At the same time, BAZ2A primes rRNA silencing by mediating the interaction between NoRC and Polymerase I promoter (Pol1). In particular, BAZ2A engages the promoter-proximal terminator T_0 of polymerase I (Pol1) through the transcription terminator factor (TTF-I). Subsequently, NoRC binds to the Sin3 corepressor complex, promoting nucleosome deacetylation at the level of rDNA^[169]. In addition, a more recent study highlighted a new BAZ2A's role in maintaining genome stability by tightly regulating perinucleolar heterochromatin formation^[170].

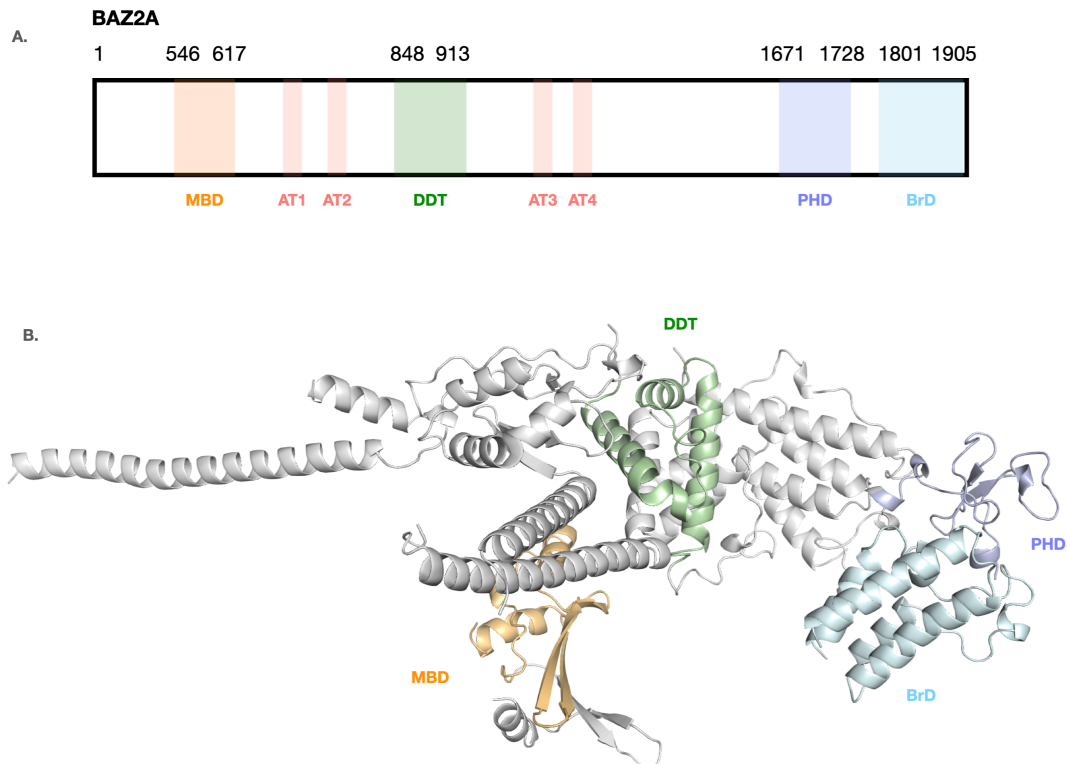


Figure 1.15: BAZ2A Domain Organization - Panel A reports the 2D illustration of human BAZ2A domain organization. Various domains are depicted in different colours. Figure adapted from Tallant et al^[165]. Panel B reports the predicted BAZ2A AlphaFold3 model, the different domains are illustrated in different colours.

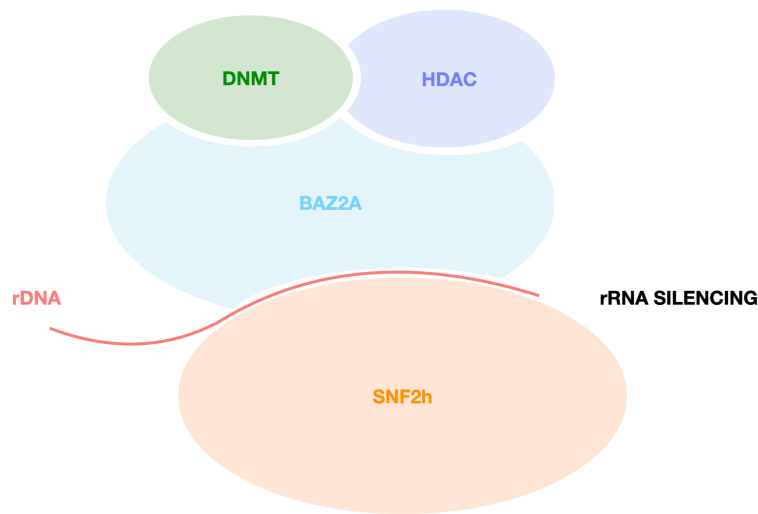


Figure 1.16: NoRC Complex in Action - Simplified representation of NoRC complex mediating rRNA silencing. The triggering event consists of Kac recognition by the BAZ2A subunit, subsequently recruiting SNF2h, DNMT and HDAC proteins involved in mediating heterochromatin establishment and rRNA silencing. Figure adapted from Postepska-Igielska & Grummt^[171].

Structural characterization combined with biophysical assays of BAZ2A Brd and PHD domains further elucidated their interactions with histone proteins, fundamental in driving rRNA silencing. Studies showed a stronger BrD preferential binding for H4K16ac, as expected, and H3K14ac residues, highlighting a tendency to recognize the KacXXR pattern. Meanwhile, the PHD domain established interactions with unmodified H3K4 tails. These findings underscore the complementary roles of the BrD and PHD domains in recognizing distinct histone marks, contributing to the regulatory mechanism of rRNA silencing^[165].

1.2.5 BAZ2A: A Crucial Driver in the Progression of Late-Stage Prostate Cancer

Prostate cancer arises from a complex combination of factors, including both genetic and epigenetic aberrations. However, the reduced number of somatic mutations occurring in prostate cancer suggested a predominant role of epigenetic pathways in promoting tumorigenesis^{[172][173]}. In this context, researchers detected relevant overexpression of BAZ2A in prostate cancer by exploiting the Brase dataset, composed of 51 and 48 tumours and normal samples, respectively^[174]. Thus, BAZ2A was inserted in the high-ranking category alongside well-known epigenetic regulators in cancer, like EZH2, CHD6 or PHF12. Scientists further proved BAZ2A overexpression even in early-onset and metastatic prostate cancer samples, noticing concurrent upregulation also of EZH2. The reason behind BAZ2A overexpression in cancer should be ascribed to the loss of miR-133a, which interacts with 3' UTR of BAZ2A and was found downregulated in the analyzed prostate cancer samples^{[175][176]}. However, previous data linking BAZ2A to cell cultures reported opposite results, demonstrating that BAZ2A depletion promoted rDNA transcription and increased cell proliferation in NIH3T3 and COS cell lines^[177].

In contrast, BAZ2A knockdown via siRNA exhibited an anti-proliferative effect in metastatic prostate cancer cell lines. This paradoxical upregulation of BAZ2A in prostate cancer suggests a broader role for the protein, potentially extending beyond its classical function in rRNA transcription inhibition. In addition, researchers observed impaired migration and invasion properties upon BAZ2A ablation in metastatic cells (Figure 1.17). Therefore, BAZ2A's pathological function relates to metastatic and aggressive features of late-stage cancer. Due to the paradoxical role of BAZ2A in prostate cancer, scientists investigated additional genes under BAZ2A abnormal regulation by performing microarray on PC3 (metastatic prostate cancer cell line) depleted of BAZ2A and EZH2. This analysis indicated 1921 and 1793 dysregulated genes upon BAZ2A and EZH2 ablation, revealing approximately one-third of abnormally expressed genes under the control of both proteins (Figure 1.18). Thus, BAZ2A collaborates with EZH2 to control several pathways involved in prostate cancer aggressiveness. Notably, gene set enrichment analysis revealed a correlation between dysregulated genes and metastatic (extracellular matrix or collagen formation) or immune pathways (cytokine and interferon signalling). In addition, unusual BAZ2A localization in the nucleoplasmic region of PC3 cells further proved the aberrant role of the protein in cancer conditions. Due to the direct interaction of BAZ2A with DNMT, researchers investigated the methylation state when BAZ2A upregulation occurred. Analyses revealed elevated levels of DNA methylation correlating to late-stage prostate cancer. In particular, 27 genes presented abnormal hypermethylation upon BAZ2A upregulation while showing increased expression in PC3 cells under BAZ2A and EZH2 elimination. Remarkably, genes involved in hypermethylation were associated with relevant pathways in tumorigenesis, such as cell growth or genome stability.

To conclude, scientists investigated the putative role of BAZ2A as a predictor of tumour relapsing. In detail, they took into account 7682 informative samples, discovering high levels of BAZ2A in late-stage cases using immunostaining. Then, they exploited Cox regression multivariate analysis to determine BAZ2A prediction power, finding that BAZ2A levels were a good indicator of cancer relapsing, independently from PSA (Prostate-specific antigen)^[176].

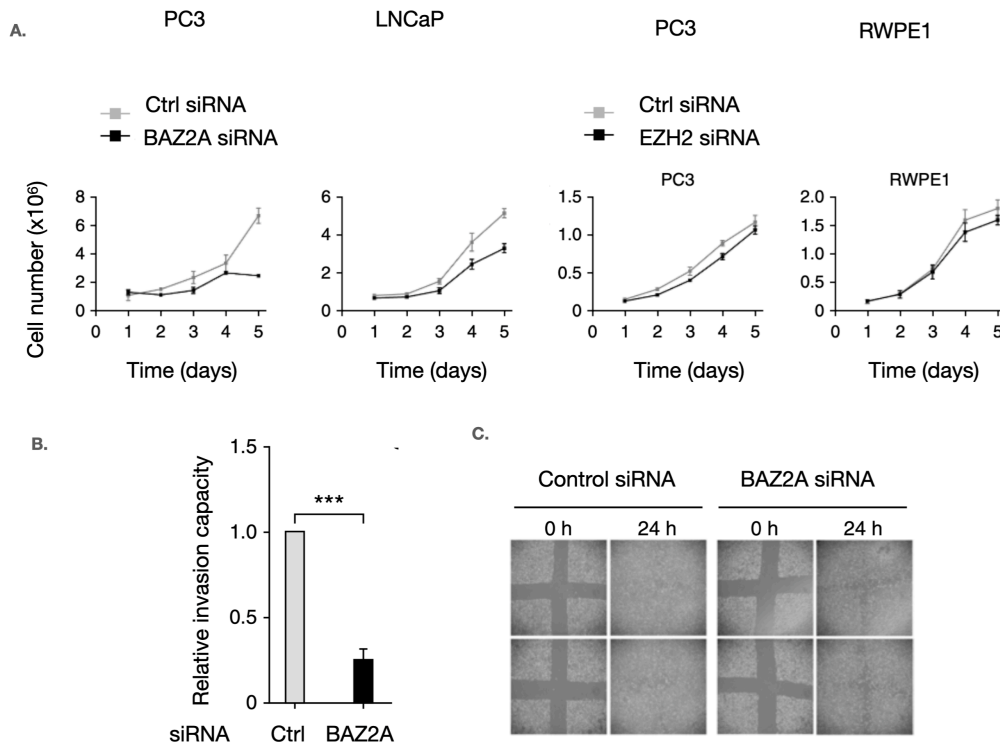


Figure 1.17: *BAZ2A* mediates metastatic and aggressive features in late-stage prostate cancer - Panel **A** reports cell proliferation curves of PC3, LNCaP and RWPE1 prostate cancer cell lines treated with siBAZ2A and siEZH2. Results show an antiproliferative effect upon siBAZ2A treatment. Panel **B** illustrates the results of the Boyden Chamber assay, showing a reduced invasion capacity when BAZ2A is ablated by siRNA. Panel **C** illustrates the results of a wound healing assay, indicating reduced migration ability upon BAZ2A elimination. Figure from Gu et al^[176].

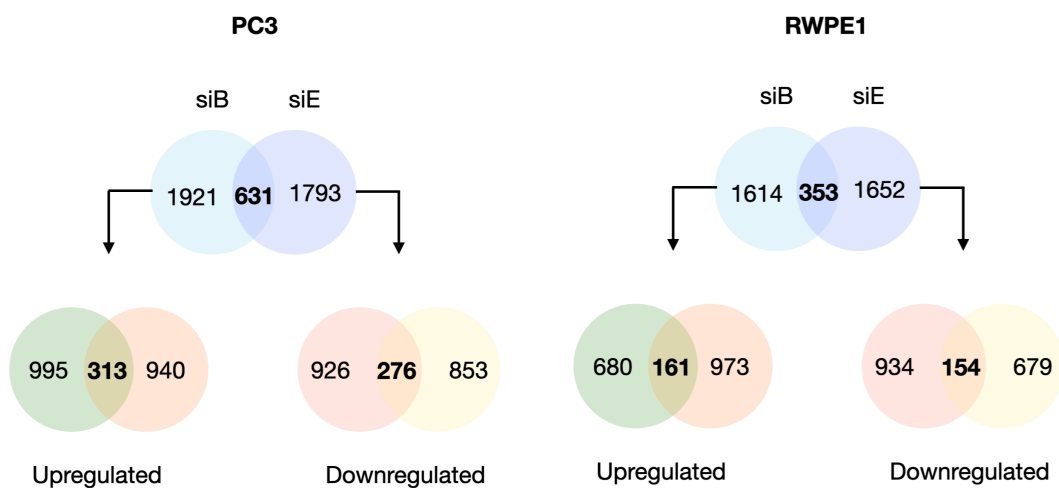


Figure 1.18: Gene deregulation under BAZ2A and EZH2 control - Venn diagrams of BAZ2A- and EZH2-regulated genes in PC3 and RWPE1 cell lines. Upregulated and downregulated genes are reported below. Figure adapted from Gu et al^[176].

The necessity to find early-stage prostate cancer biomarkers detecting tumour aggressiveness combined with the complex landscape initiating tumorigenesis highlights the urge to determine the molecular underpinnings of prostate cancer^{[178][179]}. In this context, the tissue responsible for the tumour origination could predict future scenarios related to cancer aggressiveness; several studies speculated that cancers arising from luminal tissue correlated with poor disease outcomes^{[180][181]}. Notably, a recent study linked BAZ2A's pathological role to tumorigenic transformation in luminal cells, identifying this protein as a fundamental player in prompting tumorigenic transformation determined by PTEN-loss, showing a more negative impact on the luminal cell compartment commonly associated with aggressive cancer forms^[182].

The latest article published in 2021 reported a new pathological role of BAZ2A in promoting cancer stem cell traits^[183]. The heterogeneous population composing tumours derives from a pool of undifferentiated cells, giving rise to different cellular lineages and undergoing self-renewal^[184]. Such a population is known as cancer stem cells (CSCs) and is responsible for therapeutic resistance, immune evasion, cancer spreading and reoccurrence^{[185][186]}. Prostate cancer exhibits a high level of heterogeneity that contributes to difficult diagnosis and treatment. Notably, several studies identify CSCs as responsible for this complex scenario, suggesting a new strategy for cancer treatment by targeting this pool of cells. In this context, researchers tested tumorsphere formation derived from PC3 cells in the presence and absence of BAZ2A. Results revealed that all the lines treated with shRNA-BAZ2A did not display the formation of tumorspheres, suggesting the pivotal role of BAZ2A in mediating stem cell-like features. In addition, the mutant BAZ2A BrD version abolished tumorsphere formation, demonstrating that functional BrD is necessary for CSCs properties. Therefore, the authors hypothesized that BAZ2A-BrD pharmacological inhibition could impair the CSC pool. To test their assumption, scientists treated PC3 with known BAZ2A BrD inhibitors since the beginning and observed a drastic reduction in tumorsphere formation, independently from BAZ2A expression levels (Figure 1.19). In summary, the authors uncovered BAZ2A's function in promoting stem cell properties, proposing BAZ2A-BrD inhibition as a novel strategy to impair CSCs' tumorigenic features^[183].

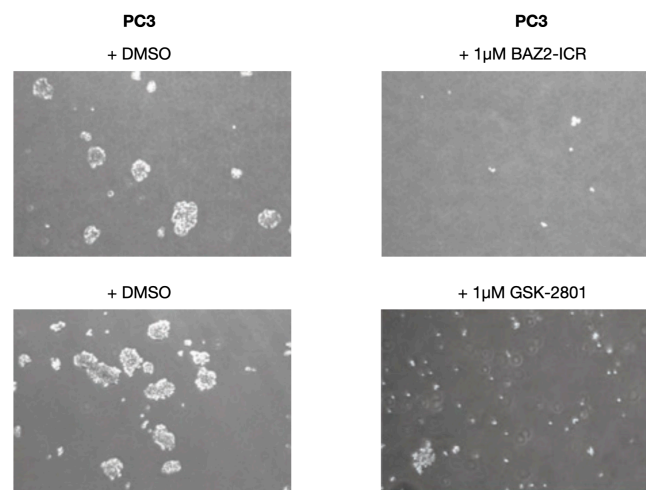


Figure 1.19: Tumorspheres formation upon BAZ2A inhibitors treatment - Representative images of tumorspheres generated from untreated PC3 cells and treated with BAZ2-ICR (1 μ M) or GSK2801 (1 μ M). Tumorsphere formation is impaired by BAZ2A inhibitors treatment. Figure from Peña-Hernández et al^[183].

In conclusion, extensive studies on BAZ2A's role in prostate cancer revealed various features

related to tumour aggressiveness, invasion, and migration abilities^[176]. Additionally, this protein plays a crucial role in enabling PTEN-deletion transformation in the luminal region and promoting stem cell oncogenic properties^{[182][183]}. Collectively, these results unveil BAZ2A targeting as a new type of intervention to counteract prostate cancer aggressiveness and impair the CSC pool tumorigenic activity.

1.2.6 Structural and Functional Insights into BAZ2B Protein

BAZ2B (BRD adjacent to zinc finger domain protein 2B), the BAZ2A paralog gene, is a 240 kDa large protein sharing 29% sequence identity with the related BAZ2A. Such similarity reaches up to 50% homology in the PHD and BrD regions, resulting in a similar domain organization (Figure 1.20)^[165].

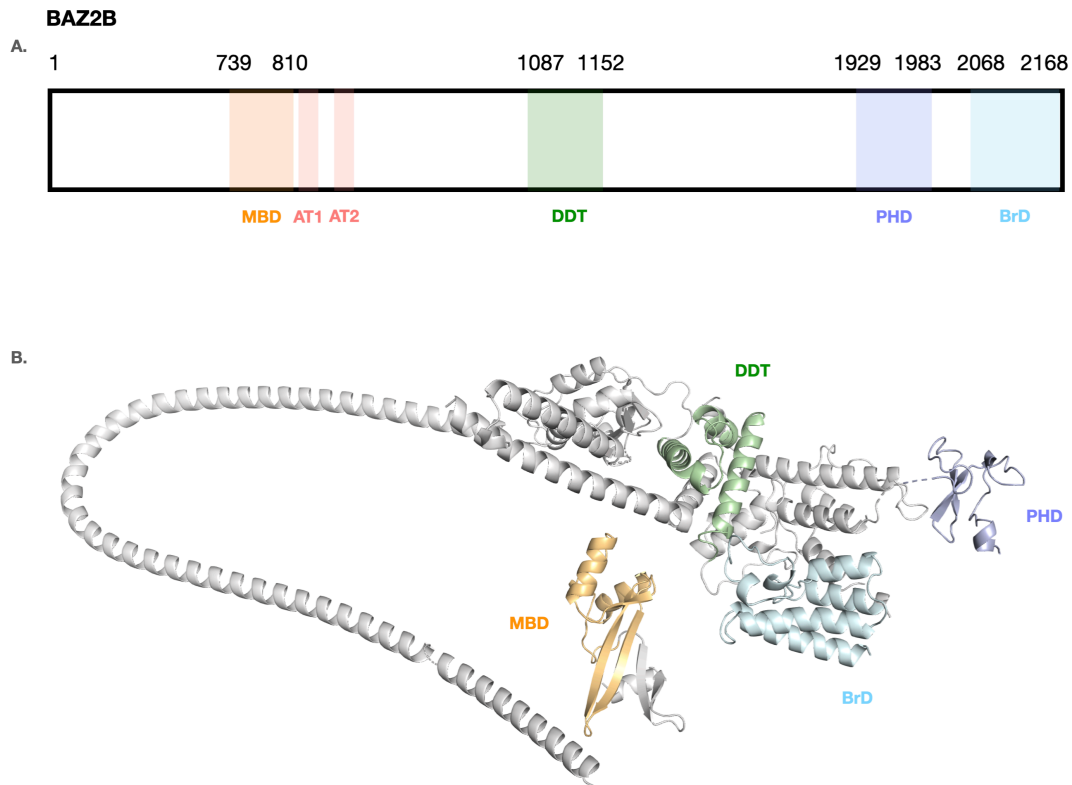


Figure 1.20: BAZ2B Domain Organization - panel **A** reports the 2D illustration of human BAZ2B domain organization. Various domains are depicted in different colours. Figure from adapted from Breindl et al^[190]. Panel **B** reports the predicted BAZ2BA_{AlphaFold3} model, the different domains are illustrated in different colours.

Since 1999, when scientists characterized the BAZ family for the first time, the role of BAZ2B remains elusive^[187]. A recent study linked BAZ2B function to nucleosome organization and mobilization. Similarly to BAZ2A, BAZ2B is part of the ISWI chromatin remodeling complex, which usually controls DNA accessibility by nucleosome sliding. In detail, the ISWI family harbours an ATPase subunit, either SNF2L or SNF2H, with an accessory subunit. In this study, scientists added to the class of accessory subunit also BAZ2B. By a series of experiments, researchers observed that ISWI complexes formed by BAZ2B and either SNF2L or SNF2H presented the propensity to mobilize nucleosomes toward the centre^[188].

In structural terms, the BAZ2B bromodomain shares multiple features with BAZ2A, recognizing mainly acH4K16 residue. Similarly to the paralog, BAZ2B BrD displays a significant affinity for acetylated residues in H3, showing a preferential binding ability to Kac-X-X-R sequences characterized by hydrophobic or aromatic residue in the second X position. Thus, such a complex level of specificity in Kac recognition further proves the BAZ2B role in prompting chromatin remodelling upon Kac identification^[165]. An additional study investigated the effect

of secondary structure on histone recognition. Researchers showed that the H3 tail helical fold is essential for the recognition exerted by BAZ2A/B-PHD domains. They proposed that an acidic region (Asp/Glu residues) in the PHD domain stabilizes H3 helical conformation through electrostatic interactions with a positive dipole region in the histone tail. In addition, the authors found 36 human PHD featuring the same consensus sequence, confirming the relevance of secondary structure recognition for chromatin remodeling complexes^[189].

Recent research has examined BAZ2B's nucleic acid binding properties, revealing a domain organization nearly identical to that of BAZ2A. Proximally to MBD, they found two additional AT-hook domains devoted to DNA/RNA recognition. Notably, molecular dynamics simulations indicated that the MBD domain with AT-hook motifs presented a weak affinity for nucleic acids, suggesting a more regulatory role of these regions. In addition, they found that MBD and AT domains exhibited high binding affinity for single-stranded DNA, proposing a putative BAZ2B role in telomere maintenance, DNA replication/repair, or DNA protection from nuclease activity. In the future, additional insights on MBD/ATM features will clarify the complex function of BAZ2B in chromatin remodeling^[190].

Some hints on the BAZ2B function also came from pathological conditions related to this protein. In the first paper linking BAZ2B to pathology, the authors conducted genome-wide association analysis (GWAS) on individuals experiencing sudden cardiac death (SCD). The study revealed that BAZ2B was associated with SCD, indicating the relevance of this protein as a risk allele for SCD^[191].

Initial studies associated BAZ2B with autism spectrum disorder, detecting high levels of this protein concurrently with other related genes^[192]. Therefore, scientists noticed a considerable number of loss-of-function BAZ2B variants in individuals with intellectual disabilities^[193]. The latest article reported ten new unrelated cases presenting deleterious heterozygous BAZ2B variants. Notably, they confirmed that autosomal dominant neurodevelopment syndrome featuring intellectual disabilities depended on BAZ2B haploinsufficiency^[194].

The latest evidence connecting BAZ2B to pathological conditions unveiled a new role in Alzheimer's disease (AD) progression. By exploiting the *C. elegans* model of age-related decline, scientists detected 59 screening hits through genome-wide RNA interference. Further analysis revealed two main hits acting as primary nodes in the network, corresponding to BAZ-2 and SET-6. The former is the orthologue of human BAZ2A and BAZ2B, while the latter performs H3K9 methyltransferase activity. Therefore, the authors deleted the BAZ-2 and SET-6 expressions in worms and observed the ageing process. Experiments showed that BAZ-2 or SET-6 ablation slowed age-related behavioural decline, while young worms were unaffected. Then, scientists discovered that BAZ-2 and SET-6 ablation did not show an additive effect, indicating that these proteins belong to the same pathway controlling H3K9 methylation in *C. elegans*.

Additional analysis revealed that BAZ-2 and SET-6 simultaneously occupied the promoters of 2383 genes, a large part encoding ribosomal and mitochondrial proteins. Thus, BAZ-2 and SET-6 combinatorial regulation of H3K9 methylation mediated the silencing of genes involved in mitochondrial functions. To confirm this hypothesis, researchers measured mitochondrial activities, like ATP production and oxygen consumption, upon BAZ-2 or SET-6 upregulation and downregulation. Results revealed that BAZ-2 and SET-6 collaborate in repressing mitochondrial protein expression, consequently promoting the ageing process. As anticipated, the BAZ-2 corresponding human homologous are BAZ2A and BAZ2B proteins. In particular, researchers detected increasing BAZ2B expression during ageing by analyzing human prefrontal cortex samples. Mouse primary cultures ablated of BAZ2B confirmed the previous observations, displaying increased levels of mitochondrial proteins. Finally, the authors constructed null BAZ2B (BAZB^{-/-}) mice, detecting improved mitochondrial activity in the hippocampal and cerebellar regions of 12-month-old animals. In addition, BAZ2B elimination avoided weight gain

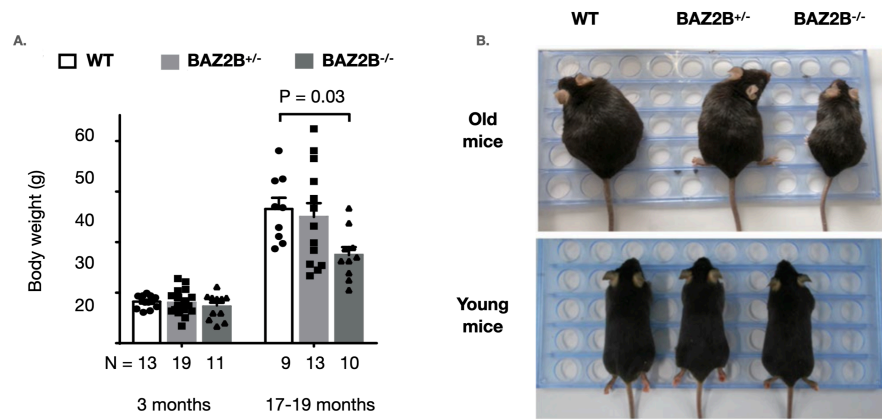


Figure 1.21: *BAZ2B* Depletion - Panel A illustrates the body weight of old and young WT, *BAZ2B*^{+/-}, and *BAZ2B*^{-/-} mice. The number of tested mice is under the bars chart. Panel B is reporting representative pictures of old and young wild-type, *BAZ2B*^{+/-} and *BAZ2B*^{-/-} male mice. Figure from Yuan et al ^[195].

mediated by ageing in male mice (Figure 1.21).

In conclusion, these results indicated that *BAZ2B* overexpression promotes the ageing process by impairing mitochondrial functioning. Notably, AD human datasets reported increased expression of *BAZ2B* genes in the prefrontal cortex, demonstrating this protein's deleterious function when overexpressed^[195].

1.2.7 Targeting BAZ2A and BAZ2B: Current Inhibitors and Biological Impact

Recent studies linking BAZ2A/B to prostate cancer aggressiveness and AD progression highlighted the urge to design new potent compounds impairing their pathological functions^{[176][195]}. However, developing a selective BAZ2A/B inhibitor proved difficult due to the structural conformation of their bromodomains. The latter holds a shallow pocket that hardly accommodates a chemical compound establishing relevant interactions with the protein.

One of the first remarkable works reporting the discovery of fragments interacting with BAZ2B dates back to 2013. In the article, scientists employed a combination of biophysical fragment screening and structure-based optimization, leading to the development of a series of hit compounds interacting in the micromolar range with BAZ2B^[196].

Later, scientists designed a potent inhibitor holding a nanomolar affinity for the targets. This compound, named BAZ2-ICR, derived from an initial virtual screening campaign that ended with a small molecule weakly binding to the targets. The co-crystal structure of BAZ2B with this first hit revealed an unexpected binding pose, arranged in a packed conformation determined by an intramolecular π -stacking between an aromatic ring and a triazole moiety. In addition, Kac's pocket was predominantly occupied by a phenyl ring, establishing hydrophobic interactions in the binding site. This feature prompted researchers to perform structural modifications involving the phenyl ring substitution with a pyrazole moiety. The replacement led to approximately 100-fold increased target affinity by mimicking the Kac binding modality (Figure 1.22). Additional structural changes in other regions led to the development of the final form of BAZ2-ICR. Isothermal titration calorimetry (ITC) experiments showed a K_d of 109 nM and 170 nM of BAZ2-ICR for BAZ2A and BAZ2B, respectively (Figure 1.23). In addition, BAZ2-ICR presented only a mild engagement to CECR2 bromodomain and did not show any other off-target activity^[197].

Similarly to BAZ2-ICR, the GSK-2801 discovery started with small fragments weakly interacting with the targets and mimicking Kac binding. Among the different molecules, the indolizine moiety displayed a 1.5 μ M affinity for BAZ2A, encouraging researchers to design a new chemical probe starting from the indolizine structure. By modelling this molecule into the BAZ2B pocket, scientists planned a series of structural changes that led to the design of GSK-2801. In particular, the most relevant chemical modifications involved the phenyl ring region functionalized by a 2-hydroxyl-methyl group. The introduction of this moiety generated a rotation at the aromatic ring level that did not fit BET pockets (Figure 1.24). Although scientists spent considerable effort in GSK-2801 selectivity to BAZ2A/B bromodomains, this compound exhibited a relevant affinity for other BrDs, especially for BRD9 and TAF1(L). Nonetheless, GSK-2801 is one of the most potent molecules targeting BAZ2A and BAZ2B bromodomains, presenting a 260 nM and 140 nM affinity for BAZ2A and BAZ2B, respectively^[198].

The latest studies investigating new probes targeting BAZ2A and BAZ2B reported the development of two hit compounds engaging their bromodomains. The first molecule originated from the connection of two fragments. In the first step, scientists performed high-throughput docking on the Maybridge Screening Collection and Enamine Golden Fragment libraries. The screening results suggested 16 putative BAZ ligands. However, co-crystallization attempts of the predicted inhibitors with BAZ2A produced well-diffracting crystals only with one compound. Therefore, the authors exploited these samples to perform back-soaking experiments with additional fragments. Surprisingly, some structures presented the simultaneous binding of the initial compound and another fragment, suggesting structural modifications based on the conjunction of the two moieties. By joining the two fragments and conducting further chemical changes, researchers ended up with hit compound **21**, which efficiently engaged BAZ2A with 10 μ M affinity (measured by AlphaScreen). In terms of structure, compound **21** formed a direct hydrogen bond between Trp1816 and the pyridine ring, deviating from the binding pose observed in the par-

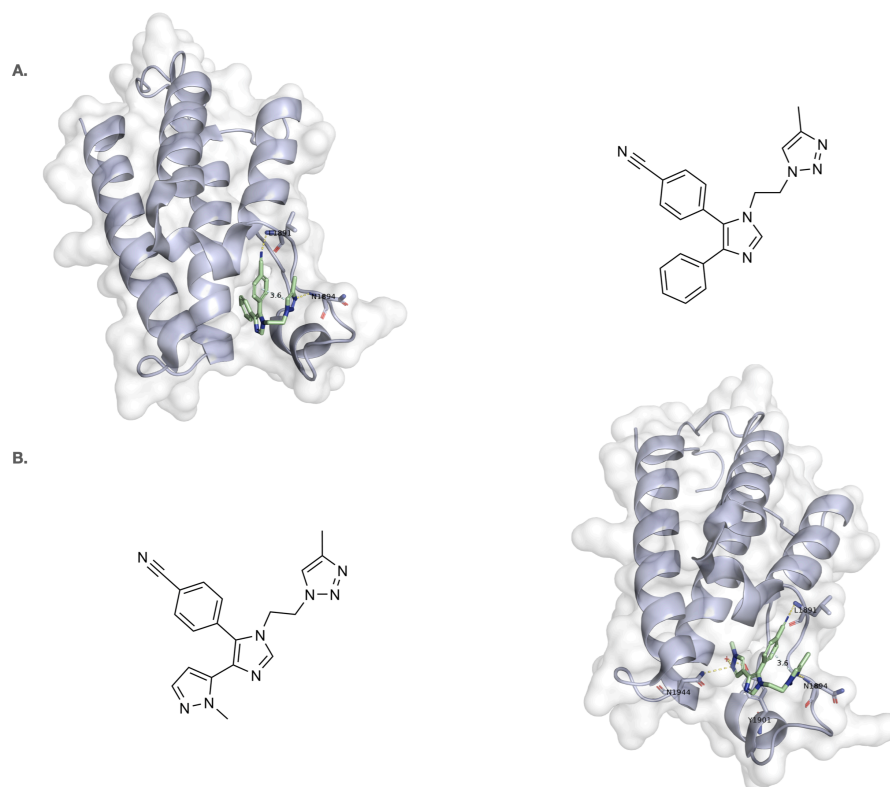


Figure 1.22: BAZ2-ICR Parent Compounds - Ribbon representation of BAZ2B bromodomains in complex with BAZ2-ICR parent compounds (PDB 4XUA; PDB 4XUB); alongside are reported BAZ2-ICR parent compounds chemical structures.

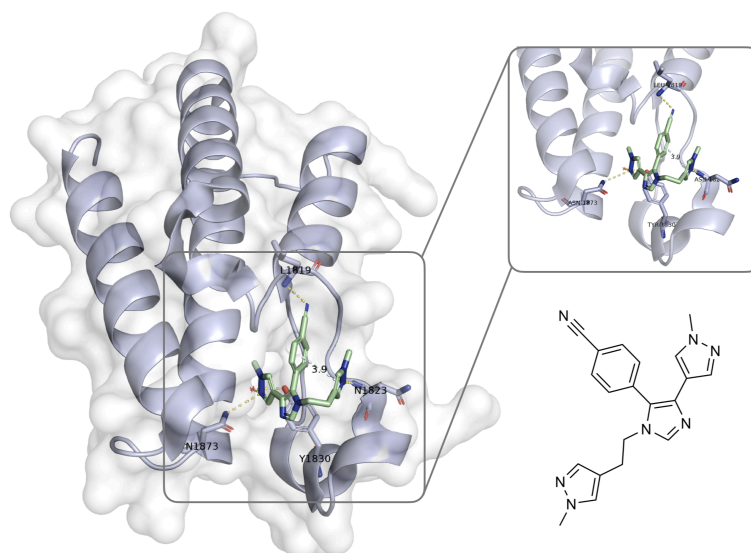


Figure 1.23: BAZ2-ICR Inhibitor - Ribbon diagram of the BAZ2-ICR probe bound to BAZ2A bromodomain (PDB 7BL8), the chemical structure of the inhibitor is reported alongside.

ent compounds. As expected, triazole moiety recapitulated Kac standard hydrogen bonds with Asn1873 and Tyr1830 residues. At the same time, compound **21** interacted with the Asn1823 main chain, like GSK-2801, while engaging Trp1816 through π - π stacking as BAZ2-ICR (Figure

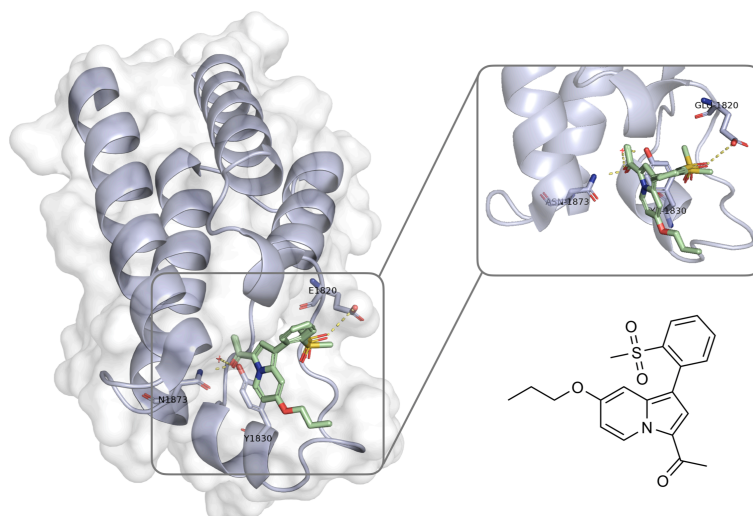


Figure 1.24: GSK-2801 Inhibitor - Ribbon representation of the GSK-2801 probe bound to BAZ2A bromodomain (PDB 7BL9), the chemical structure of the inhibitor is reported alongside.

1.25)^[199].

As anticipated, scientists exploited a similar procedure for another BAZ2A/B hit compound discovery. Similarly to compound **21**, researchers conducted an extensive virtual screening, resulting in 80 positive hits. By combining AlphaScreen results with crystal structures, researchers envisioned compound **104** presenting a 4 μ M affinity for BAZ2A (measured through AlphaScreen). Structural characterization revealed that the acetyl pyrrole unit formed conserved hydrogen bonds with Tyr1830 and Asn1873 while interacting with the Pro1817 main chain. The higher affinity for the target was due to an additional hydrogen bond between the ethyl-isoxazole tail and the Asn1823 main chain (Figure 1.26, 1.27) ^[200].

Both compounds **21** and **104** represent a good starting point for the development of BAZ2A/B inhibitors in the future. High-resolution crystallographic structures revealed that, for both compounds, a single enantiomer binds specifically to the Kac pocket, despite co-crystallization experiments and affinity measurements being conducted with racemic mixtures. Although the binding affinities observed were relatively low, enantiospecificity could be significant for enhancing targets affinity, potentially offering a valuable route to design more targeted and selective inhibitors^{[199][200]}.

BAZ2A and BAZ2B inhibition showed promising results in different pathologies, including triple-negative breast cancer (TNBC) and inflammatory bowel disease^{[201][202]}. The former is a complex form of breast cancer featuring the absence of three principal receptors: estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Thus, TNBC patients undergo non-targeted treatment and display a higher risk of tumour relapsing. Consequently, further investigations on TBNC molecular drivers and new therapeutical interventions are urgently requested^[203]. In this context, co-treatment of TBNC cell lines with JQ1 and GSK-2801 resulted in an anti-proliferative effect. In particular, the combinatorial administration of these inhibitors produced a synergistic effect, suggesting a concurrent impairment of cancer-related pathways. Notably, scientists investigated genes controlled by BET and BAZ2A/B co-inhibition, identifying TAGLN, KISS1, PTPRC, MUC19 and KCNB1 as putative molecular players in combined JQ1/GSK-2801 anticancer activity^{[201][203]}.

The latest applicability of BAZ2A/B inhibitors involves inflammatory bowel disease. Researchers

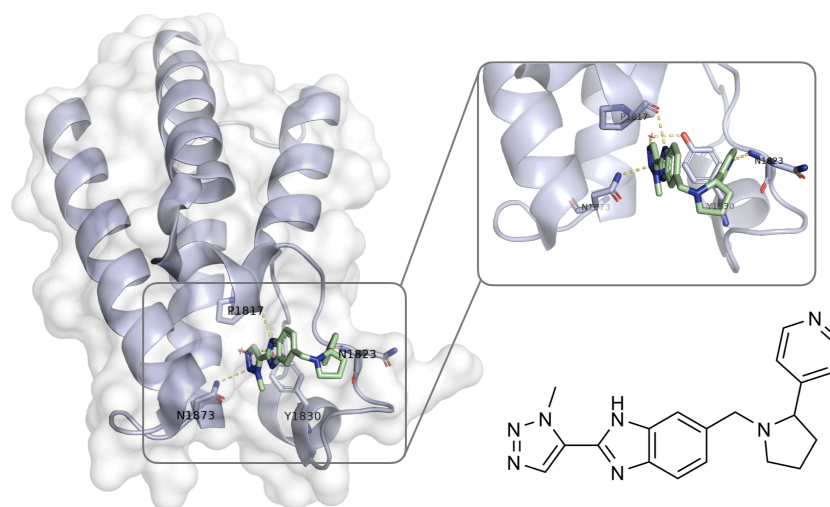


Figure 1.25: *Hit Compound 21* - Ribbon diagram of BAZ2A bromodomain in complex with compound 21, while the chemical structure of the hit-compound is reported alongside (PDB 7BC2).

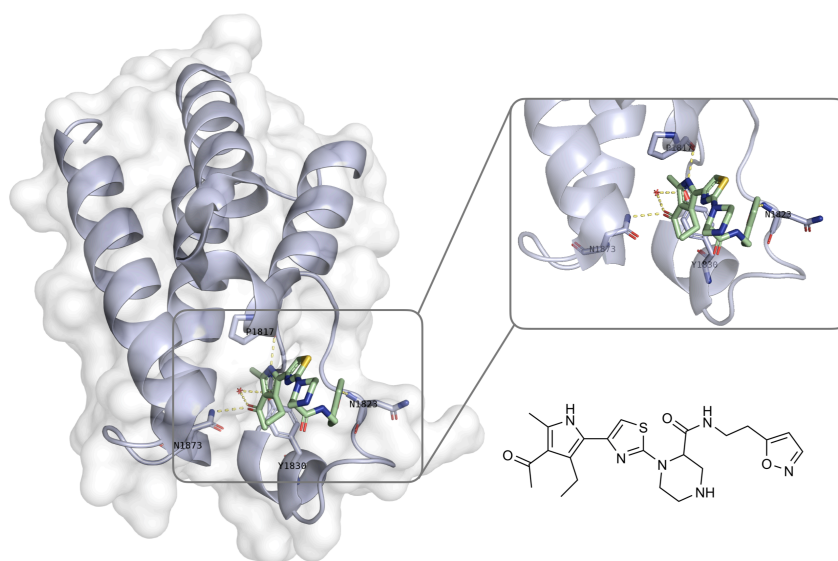


Figure 1.26: *Hit Compound 104* - Ribbon diagram of BAZ2A bromodomain in complex with compound 104, while the chemical structure of the hit-compound is reported alongside. (PDB 7QZC).

observed an increased liver regeneration ability upon BAZ2A/B inhibition in primary tissues or mice models. Notably, BAZ2A inhibition did not show aberrant cell growth, indicating the potential of BAZ2 inhibitory treatment in liver failure or transplant recovery^[202].

In summary, these results confirmed the therapeutic relevance of BAZ2A/B inhibition by targeting their bromodomain and consequently impairing Kac recognition. In the future, BAZ2 proteins inhibition strategy could find additional pharmacological applicability in prostate cancer or AD.

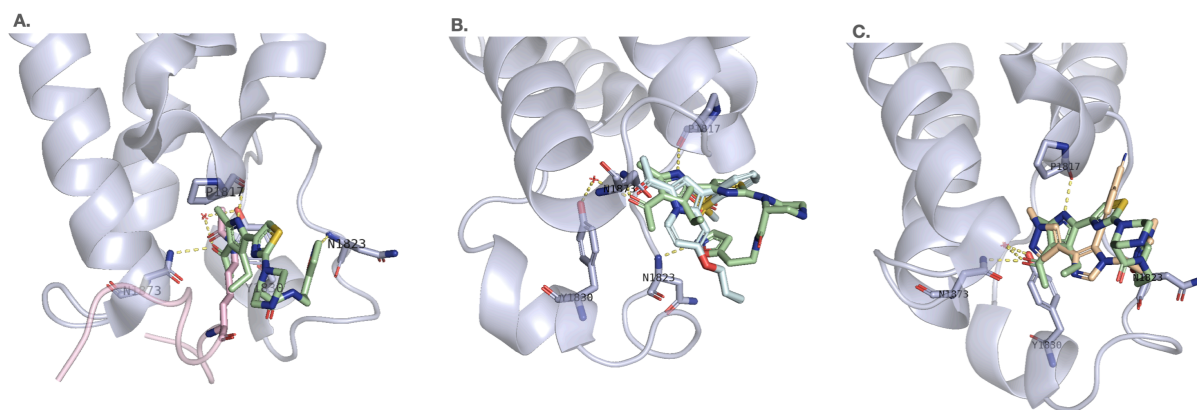


Figure 1.27: Compound 104 Binding Pose - Panel **A** illustrates the superimposition of Kac (light pink) and compound **104** (light green) in complex with BAZ2A bromodomain, showing a similar binding mode to the pocket (PDB 3UVW; PDB 7QZC). Comparably, panels **B** and **C** report the superimposed structure of BAZ2-ICR (cyan) and GSK-2801 (orange) with compound **104** in the BAZ2A binding pocket (PDB 7BL8; PDB 7BL9).

Chapter 2

Aims

Although recent investigations have linked BAZ2A to prostate cancer, no studies have explored the potential of this protein as a novel target. In this study, we assessed the possibility of inhibiting BAZ2A’s pathological function by blocking its recognition of Kac. However, testing known inhibitors on prostate cancer cell lines revealed that inhibiting the BAZ2A bromodomain is insufficient to produce an antiproliferative effect. In this context, we envisioned a novel strategy possibly promoting beneficial effects in prostate cancer cell lines, aiming to:

- Harness the power of PROTAC technology in degrading efficiently BAZ2A. By designing small molecules that conjugate a known BAZ2A bromodomain ligand with an E3 ligase recruiting moiety, we aim to target the protein for elimination rather than merely inhibition.
- Structurally characterize the ternary complexes formed by BAZ2A, the synthesized degraders, and the recruited E3 ligase. By employing X-ray crystallographic analysis, we plan to confirm target engagement *in vitro* and determine future structural modifications to improve PROTACs’ ability to form active ternary complexes.
- Evaluate the ability of our PROTACs to degrade BAZ2A in an iterative process. Testing will help identify the most effective degrader while guiding the design of new molecules with enhanced biological activity.
- Confirm the therapeutic potential of BAZ2A degradation in advanced prostate cancer, utilizing our synthesized PROTACs. Additionally, these degraders could hold promise for targeting BAZ2B, a protein implicated in ageing and Alzheimer’s disease progression, opening new possibilities for treatment across multiple conditions.

Through this innovative approach, we hope to not only shed light on BAZ2 proteins’ role in the physiological context but also propose new strategies for treating severe pathological conditions.

Chapter 3

Results and Discussions

3.1 Harnessing BAZ Proteins for Effective Prostate Cancer Treatment

Growing literature linking BAZ22A to prostate cancer encouraged us to study new possible therapeutic interventions involving the interference with this protein. As discussed in the Chapter 1.2.2, the primary strategy targeting BCP proteins acts at the level of Kac recognition. In other words, blocking the identification of specific epigenetic features strongly impaired overall protein function and has proven beneficial effects in different pathologies associated with BCP deregulation. Therefore, we hypothesized that BAZ2A's role in prostate cancer could be impaired by interfering with its ability to recognize Kac using a competitive inhibitor. In this context, we tested known BAZ2A inhibitors (BAZ2-ICR^[197] and GSK-2801^[198], more details are reported in Section 1.2.7) in a dose-response manner in prostate cancer cell lines. We opted for PC3 (androgen receptor - negative) and 22Rv1 (androgen receptor - positive) cell lines presenting different susceptibility to the androgen receptor, a known molecular driver of prostate cancer. The two inhibitors were tested at different concentrations (0.5 μ M, 2.5 μ M, 5 μ M, 10 μ M, 25 μ M) over nine days of treatment. Viability was measured every three days during the time course. Surprisingly, BAZ2-ICR did not display any effect on cell viability, while GSK-2801 slowed cell proliferation only at very high concentrations (Figure 3.1). Due to the lack of activity in PC3 cells, which plateaued between days six and nine, the final three days of treatment are not included in the curves for this cell line.

Based on these results, we hypothesized that GSK-2801 antiproliferative effect was due to the inhibition of BRD9, a known GSK-2801 target. Such protein was previously associated with different tumours, including prostate cancer, however, the ability to impair tumorigenic proliferation upon BRD9 simple inhibition is still debated^[204]. In this context, we tested a known BRD9 inhibitor (BI-9564^[205]) and evaluated its activity on PC3 and 22Rv1 cell lines. The experimental setup was similar to the previous one, cell viability was measured every three days over nine days and BI-9564 was tested at different concentrations, ranging from 0.1 μ M to 20 μ M. Treatment did not show any effect on PC3 cell line over nine days and exhibited a moderate effect on 22Rv1, which was not comparable with the one observed upon GSK-2801 treatment (Figure 3.2).

At this point, results suggested that GSK-2801 effect was not related to exclusive inhibition of either BAZ2A or BRD9, but simultaneous engagement of both proteins gave rise to a synergistic antiproliferative outcome. To assess the impact of concurrent BAZ2A and BRD9 inhibition, we tested BAZ2-ICR and BI-9564 in combination over nine days in PC3 and 22Rv1 cell lines. In particular, BAZ2-ICR concentration was maintained at 25 μ M, while BI-9564 one varied from 0.1 μ M to 20 μ M. Similar to the exclusive BI-9664 treatment, combinatorial treatment did not affect

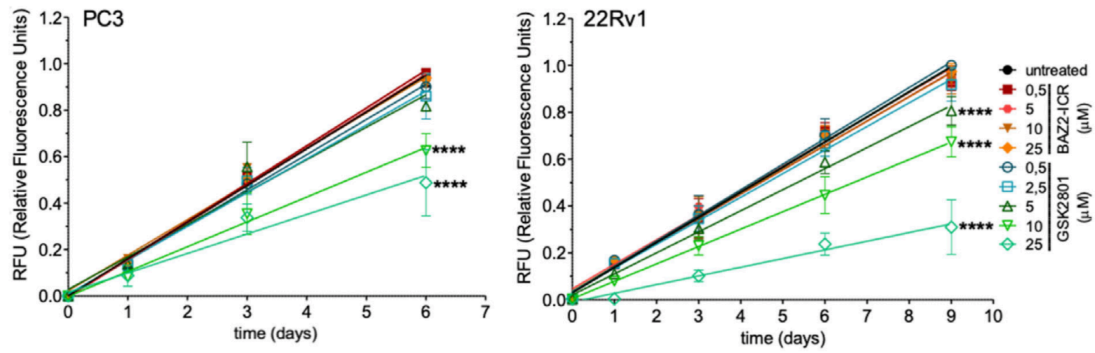


Figure 3.1: Effect of GSK2801 and BAZ2-ICR on PC3 and 22Rv1 cancer cell lines. Each point represents the mean $\pm$ SD of 3–5 independent replicates, depending on the cell lines and the treatment. The treatment was considered to have an effect when slopes for treated conditions were statistically different from the untreated. $ns = p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. Curves indicates that that GSK-2801 impaired cell proliferation at high concentrations.

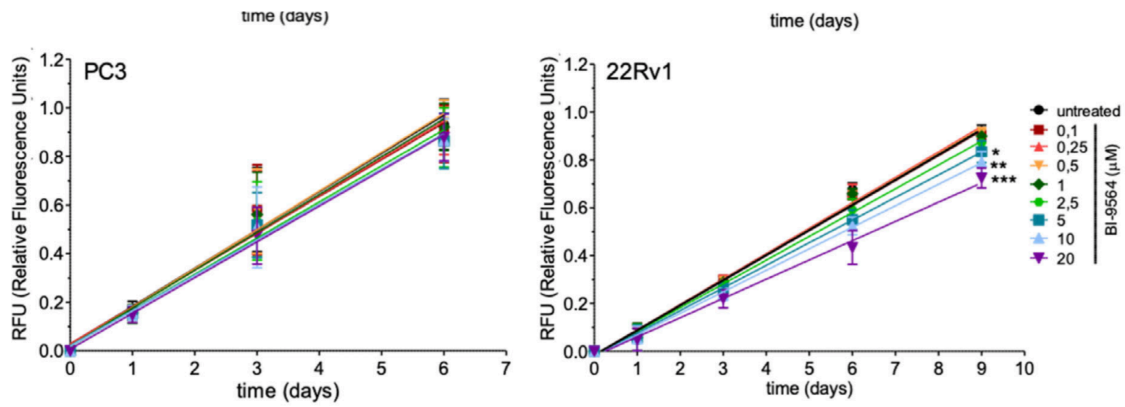


Figure 3.2: Effect of BI-9564 on PC3 and 22Rv1 cancer cell lines. Each point represents the mean $\pm$ SD of 3–5 independent replicates, depending on the cell lines and the treatment. The treatment was considered to have an effect when slopes for treated conditions were statistically different from the untreated. $ns = p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. Curves indicates that that BI-956 moderately impaired cell proliferation at high concentrations only in 22Rv1 cells, without reproducing GSK-2801 effect.

the proliferation in PC3 cells, while showing a moderate effect in 22Rv1 cells but not comparable with GSK-2801 treatment. Therefore, we proposed that GSK2801 ability to affect proliferation at high concentrations was due to additional unknown off-targets and does not relate to the simultaneous inhibition of BAZ2A and BRD9 (Figure 3.3).

Collectively, these results indicate that simple BAZ2A bromodomain inhibition is not sufficient to block the pathological function of this protein. Conversely, BAZ2A ablation through siRNA showed the ability to slow the proliferation rate in prostate cancer cell lines, as reported by Gu *et al* [176]. To better elucidate the mechanism behind BAZ2A elimination, we performed BAZ2A silencing by treating PC3 and 22Rv1 cell lines with siRNAs. As expected, ablation displayed a significant antiproliferative effect compared to untreated control. In addition, we investigated the transcription levels of genes associated with BAZ2A abnormal expression upon BAZ2-ICR inhibition or ablation (explained in detail in Section 1.2.5). Results showed that BAZ2A elimination impacted gene expression differently, further demonstrating that BAZ2A

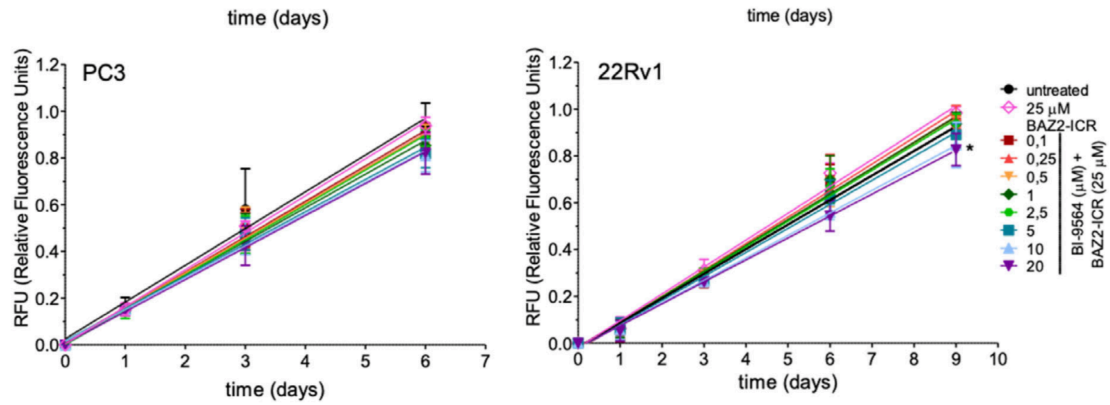


Figure 3.3: Effect of combinatorial treatment of BI-9564 and BAZ2-ICR on PC3 and 22Rv1 cancer cell lines. Each point represents the mean $\pm$ SD of 3–5 independent replicates, depending on the cell lines and the treatment. The treatment was considered to have an effect when slopes for treated conditions were statistically different from the untreated. $ns = p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. Curves indicates that that combinatorial treatment moderately impaired cell proliferation only in 22Rv1 cells, without reproducing GSL-2801 effect.

bromodomain inhibition did not recapitulate protein ablation (Figure 3.5).

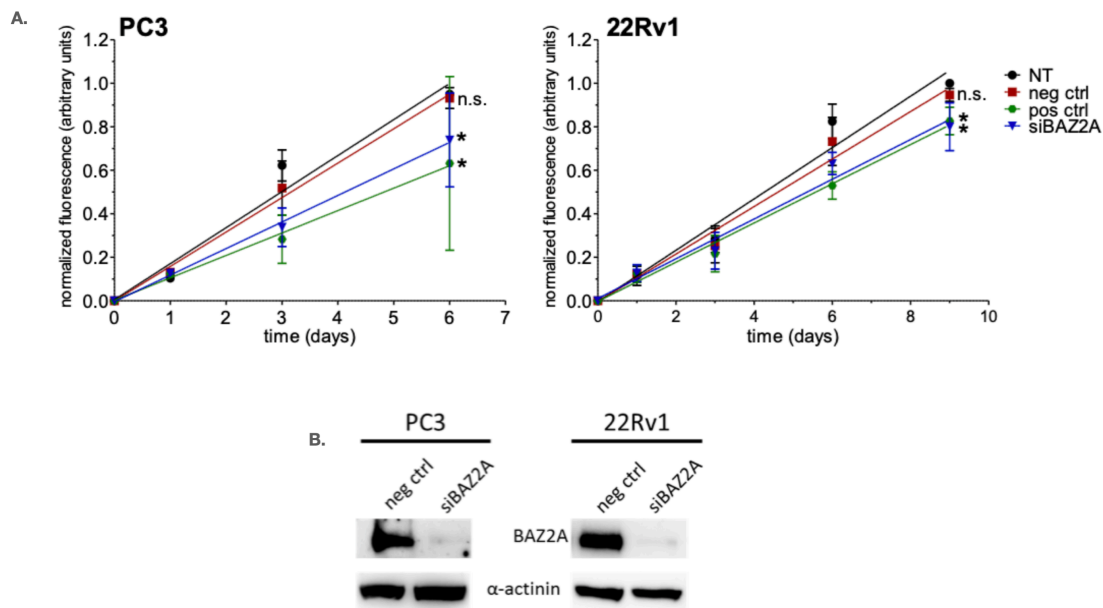


Figure 3.4: Panel **A** reports the effect of silencing BAZA through siRNA in PCa cells. The reduction of BAZ2A caused a decrease in the growth rate of both PC3 (left) and 22Rv1 (right), similar to the one observed when KIF11 (positive control) was silenced. Each point represents the mean $\pm$ SD of 3 independent replicates. The treatment was considered to have an effect when the slopes of the treated conditions and the negative control were statistically different. It was first assessed that the non-transfected condition and the negative control (transfected with siRLUC) had no significant difference. $ns = p > 0.05$, $*p < 0.05$. Panel **B** illustrates the western blot analysis of BAZ2A in prostate cancer cells. The total protein content was extracted from cells transfected with siRNA BAZ2A or siRNA RLUC (negative control). A loading control (α -actinin) was used to show that the same amount of protein lysate was loaded for the two conditions.

Collectively, our study confirmed the relevance of BAZ2A in promoting tumorigenic proliferation and showed that BAZ2A bromodomain inhibition did not phenocopy beneficial effects observed upon ablation. Therefore, we proposed a new valid strategy for targeting BAZ2A in late-stage prostate cancer consisting of degrading the entire complex through PROTAC technology. In this way, BAZ2A ablation will have an impact on prostate cancer aggressiveness reducing tumorigenic proliferation, while impairing metastatic potential and avoiding cancer spreading.

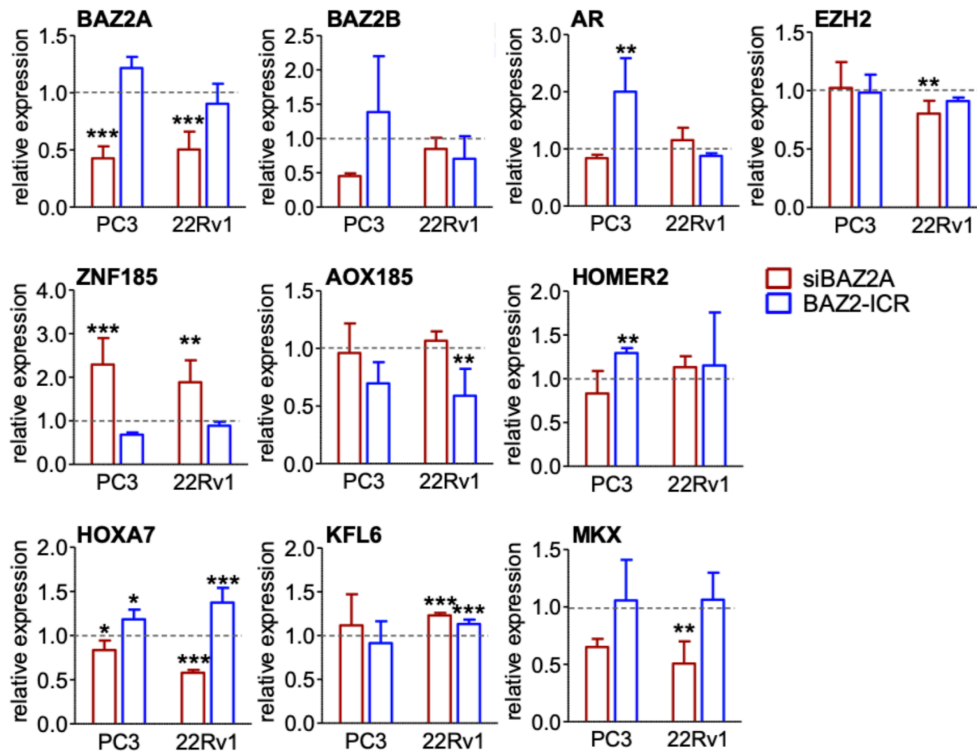


Figure 3.5: The graphs show the relative gene expression in PC3 and 22Rv1 cells of BAZ2A, BAZ2B, AR, EZH2, ZNF185, AOX185, HOMER2, HOXA7, KFL6 and MKX. The control (untreated or negative control) is represented as a grey dotted line with the value 1.0 of relative expression. The graphs show the mean $\pm$ SD of 3 independent experiments. The statistical significance was evaluated with one way Anova comparing the treatments to the respective controls. When not otherwise written, the difference resulted non-significant ($ns = p > 0.05$), $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Given the structural similarity between BAZ2A and BAZ2B bromodomains (reported in Section 1.2.6^[165]), developed BAZ2A bromodomain inhibitors often interact with a non-negligible affinity to the same domain in the BAZ2B protein. Notably, even BAZ2B ablation has proven beneficial in the ageing process, decreasing age-related weight gain and improving cognitive functions in mice models depleted of this protein^[195]. Therefore, we supposed that degraders originally envisioned for BAZ2A could have a relevant effect even for BAZ2B, by slowing the ageing progression and preventing cognitive impairment caused by neurological disorders.

These results, along with additional findings, are published in ^[206], in which I contributed as co-author.

3.2 BAZ2A/2B PROTACs Design

BAZ2A bromodomain simple inhibition proved inefficient in promoting antiproliferative effect in PC3 and 22Rv1 prostate cancer lines, demonstrating that occupying the Kac binding site did not interfere with BAZ2A pathological function. The only strategy counteracting abnormal tumorigenic proliferation occurred upon BAZ2A ablation through siRNAs. The extensive effort spent discovering selective BAZ2A/B inhibitors in our laboratory prompted us to exploit the developed compound to evolve it into a PROTAC. Notably, this new technology would allow us to reproduce the siRNA antiproliferative effect by simply administering a chemical probe derived from a previously discovered inhibitor. In addition, BCPs resulted as suitable targets for PROTAC technology, establishing relevant cooperative interactions between their bromodomain and the E3 ligase and thus promoting ternary complex formation.

The first important step to developing a BAZ2A/B PROTAC degrader, starting from a previously discovered ligand, consisted of determining the anchoring point for the linker component. To design the PROTAC degrader finely, we exploited high-resolution structures of BAZ2A bromodomain in complex with compound **104**^[200] and superimposed it with available ternary complex structures encompassing other BrD. We employed the ternary complex composed of CRBN-dBET6-BRD4 (PDB 6BOY) for the structure comparison. Notably, the dBET6 linker region resembled the same ligand pose as the piperazine moiety in compound **104**. In addition, this ring is the most exposed part in compound **104**, and the conjunction of a linker in this region will ensure a good exiting point for the other PROTAC components. In particular, piperazine moiety can better tolerate a new structural component because this will not perturb the existing interactions between BA2A/B bromodomain and compound **104**. Therefore, we hypothesized that piperazine moiety was the best anchoring region to accommodate a linker and design the first BAZ2A/B PROTAC degrader (Figure 3.6).

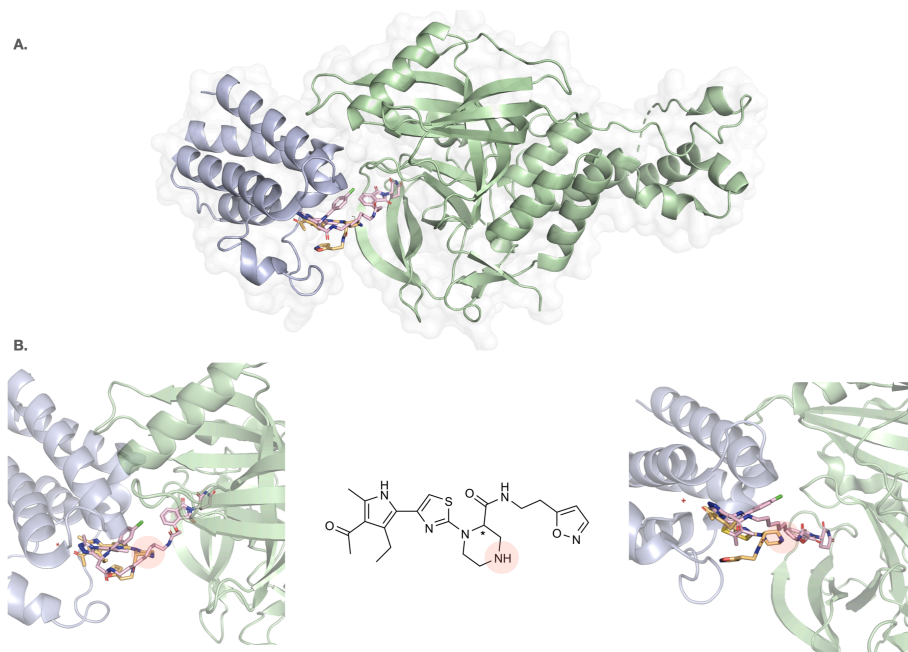
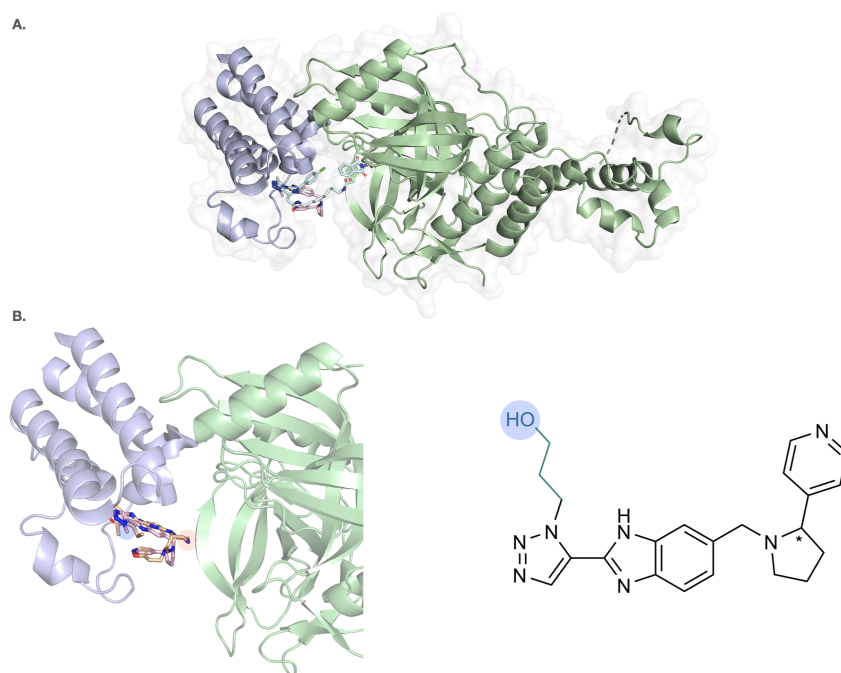


Figure 3.6: Panel **A** reports the structure superimposition of BAZ2A bromodomain bound to compound **104** (PDB 7QZC) (light blue and orange) and BRD4-dBET6-CRBN ternary complex (PDB 6BOY) (pink and light green). Panel **B** illustrates structure alignment magnification, highlighting the piperazine moiety with a red dot. In the middle of panel **B** is reported compound **104** chemical structure.

Another possible exiting point to attach linker components is represented by the ethyl substituent in the pyrrole ring of compound **104**. However, the low synthetic accessibility related to this anchoring area prompted us to cover this region by employing another previously developed hit compound named molecule **21**. Similarly to compound **104**, we superimposed the structure of compound **21**^[199] with released ternary complex structures, envisioning the exit point in the methyl substituent of the triazole ring. Thus, we tried to cover the region previously unexplored by compound **104** PROTAC, while exploiting a different BAZ2 ligand moiety. Despite compound **21** being purchased in the form of (1,2,3-triazol-1-yl)propan-1-ol, carrying a hydroxyl group as a substituent instead of a non-reactive methyl group, the synthesis of compound **21**-based PROTACs was not achieved unfortunately (Figure 3.7).



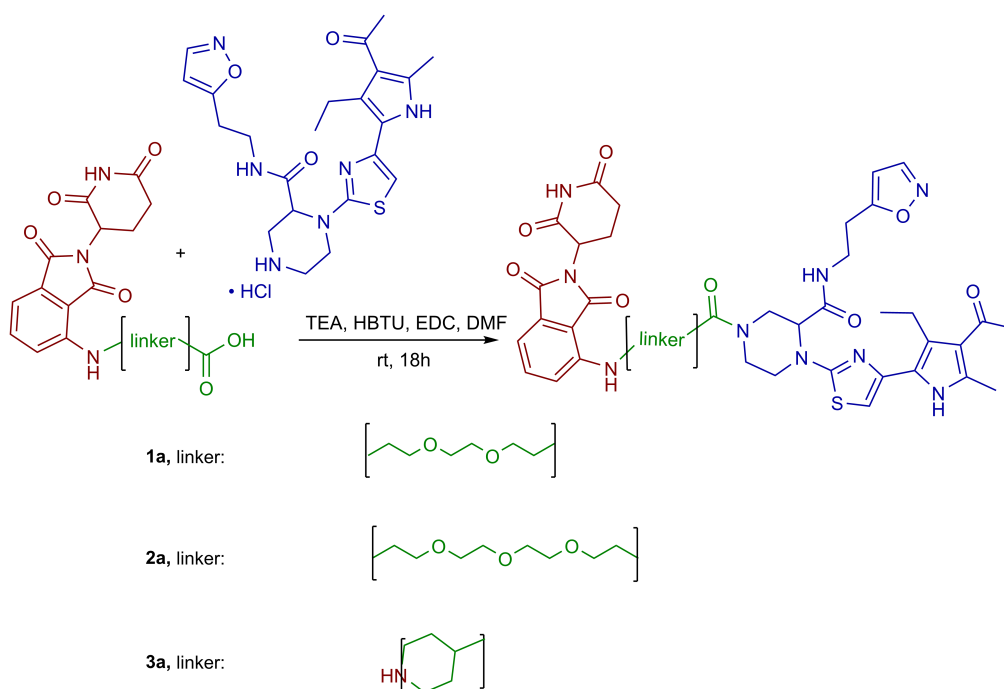
3.3 Synthesis, Chromatographic Purification and Structural Elucidation of BAZ2A/2B PROTAC Degradors

The array of synthesized PROTACs targeting BAZ2A/2B was divided into two main series: **a**, **b**. The former holds a thalidomide-like moiety devoted to CRBN E3 engagement^[207], the most commonly recruited ligase. For the latter, we opted for another well-established E3 ligase in the PROTAC field, specifically VHL^[208]. As reported in Section 3.2, we employed a previously developed hit compound in the laboratory (named compound **104**). Such molecule displayed a reduced affinity for the target mainly due to the exposed BAZ2A/B BrD pocket and the inactive enantiomeric form. However, the transient MoA of PROTAC technology ensures POI degradation regardless of target affinity, prompting us to engage BAZ2A/B by exploiting compound **104**^[200].

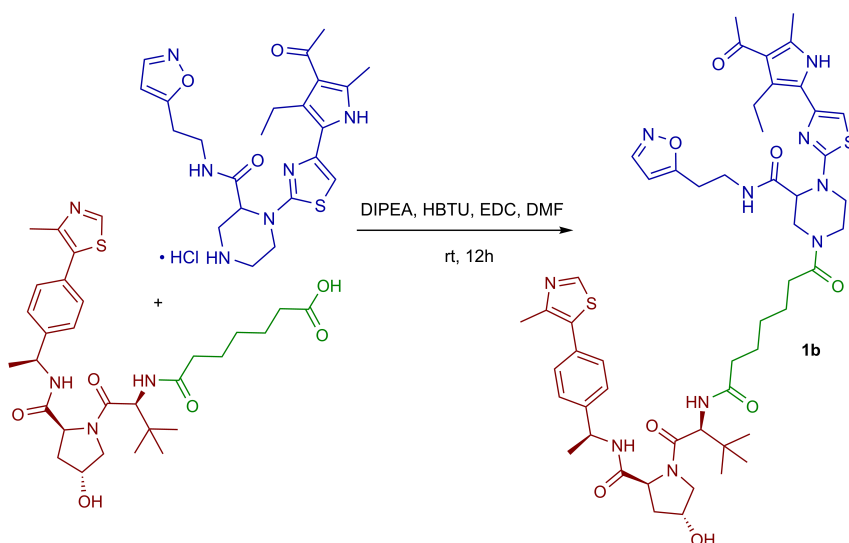
PROTACs belonging to series **a** were obtained by performing a single coupling reaction between CRBN recruiter equipped with different linkers (red and green, respectively) and compound **104**, (blue) (Figure 3.8). Both CRBN recruiter and compound **104** were purchased as racemic mixtures. The latter was dissolved in dimethylformamide (DMF) and treated with triethylamine (TEA) to free the amine group in the piperazine moiety. Then, compound **104** reacted with the carboxylic group in the linker region, forming an amide bond catalyzed by HBTU/EDC coupling. Synthesized CRBN-based PROTACs (series **a**) present three linkers: short/flexible (PEG 2 - **1a**), longer/flexible (PEG 3 - **2a**) and rigid (**3a**) (Scheme 3.8). This variability relates to the necessity to test each of our compounds iteratively, guiding the discovery of functional PROTACs with a trial-and-error approach. The first three BAZ2A/B degraders were successfully synthesized and structurally characterized by NMR spectroscopy and mass spectrometry. The purity of all final products was assessed using analytical high performance liquid chromatography (HPLC) technique, with each compound achieving a purity level higher than 95%.

The series **b** consists of PROTACs mediating degradation through VHL engagement. In this case, we exploited the VH032 ((2S,4R)-1-((S)-2-amino-3,3-dimethylbutanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide) molecule for all the synthesized degraders (**2b-8b**), apart from compound **1b** holding the VH039 version ((2S,4R)-1-((S)-2-amino-3,3-dimethylbutanoyl)-4-hydroxy-N-((S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide)^[208]. Both VHL recruiters were purchased as enantiomers. Optical activity was measured, yielding the following values: $[\alpha]_{588}^{25} = +22^{\circ}$ (5 mg/mL, methanol) ; $[\alpha]_{577}^{25} = +26^{\circ}$ (5 mg/mL, methanol) . Similarly to series **a**, **1b** synthesis consisted of a single reaction step. We maintained the same anchoring point in the POI ligand moiety (compound **104**) for the linker attachment by performing coupling reaction mediated by HBTU/EDC reagents (Scheme 3.9).

For the synthesis of **2b-7b**, we employed three reaction steps starting from the linker attachment to the E3 recruiter. First precursors (**10b**, **12b**, **14b**, **16b**, **18b**, **20b**, respectively) were synthesized by a coupling reaction between the linker carboxyl group and VH032 primary amine; the employed conditions were the same used for **1b**. We opted for linkers carrying a free carboxylic group and a protected *tert*-butyl ester, allowing the amide bond formation without generating unwanted dimeric by-products. In the second step, we removed the protecting *tert*-butyl group by an acidic treatment (trifluoroacetic acid in dichloromethane). Obtained precursors (**11b**, **13b**, **15b**, **17b**, **19b**, **21b**, respectively) underwent a second coupling reaction, allowing the attachment of compound **104** to the VHL recruiter through the linker (Scheme 3.10). Each precursors and final products were purified by either preparative thin-layer chro-



Scheme 3.8: Synthetic scheme of series **a** PROTAC degrader. CRBN recruiter is highlighted in red, while linker and POI ligand are depicted in green and blue, respectively.

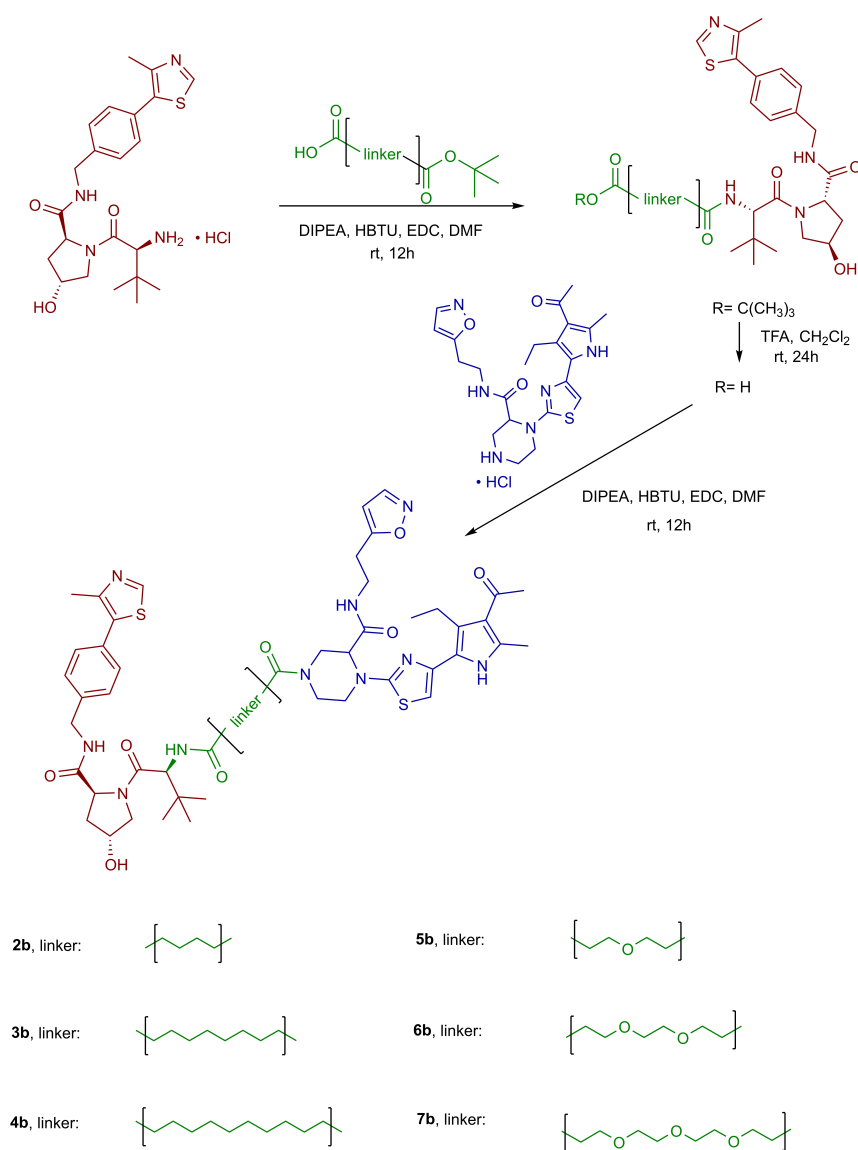


Scheme 3.9: Synthetic scheme reporting the applied procedure for compound **1b**. Various components are depicted in different colours.

matography (PLC) plate or silica column, obtaining a purity level higher than 95%, as detected by analytical HPLC analysis. In addition, structural features of the compounds were confirmed using NMR spectroscopy and mass spectrometry, as reported below.

Compounds **2b-7b** present two main linker subgroups: alkylic and peg-like chains of variable length. The idea behind using flexible structures as linkers is to allow PROTACs free exploration

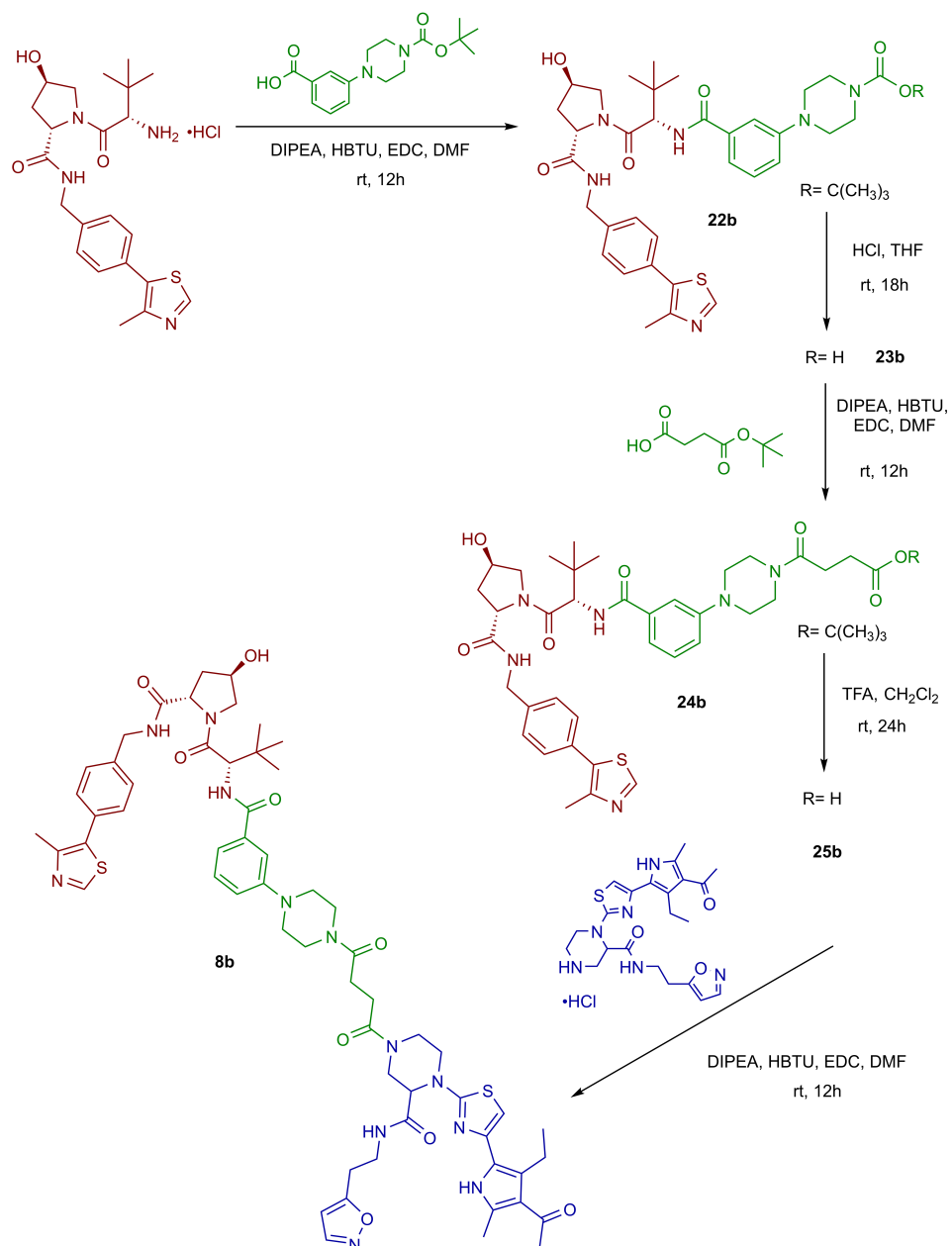
of different conformations in the interaction with the targets, and increase the chance of forming active ternary complexes.



Scheme 3.10: Synthetic scheme for compounds **2b-7b**. Various components are depicted in different colours.

On the contrary, the **8b** degrader exploits a rigid linker to maximize entropic contribution in the simultaneous binding to VHL and BAZ2A/B. The synthesis consists of five reaction steps, alternating coupling reactions to deprotections (Scheme 3.11). First, VH032 was treated with DIPEA, HBTU and EDC to favour the attachment of (*tert*-butoxycarbonyl)piperazin-1-yl)benzoic acid to the VHL recruiter to give the compound **22b**. Then, *tert*-butyl protecting group was removed under acidic conditions; standard TFA treatment did not lead to carboxylic acid conversion, while 2M HCl solution addition successfully mediated precursor **23b** formation.

In the following step, a second small linker moiety is attached to produce compound **24b**, from which the *tert*-butyl protecting group is removed by employing acidic standard conditions (trifluoroacetic acid in dichloromethane). Lastly, compound **104** was reacted with **25b** to get the final product **8b**. Similarly to the other analogues, compound **8b** was purified obtaining a purity grade > 95%, and structurally characterized by NMR spectroscopy and mass spectrometry.



Scheme 3.11: Synthetic scheme reporting the applied procedure for compound **8b**. Various components are depicted in different colours.

This work reports the synthesis, purification and structural elucidation of 11 PROTAC degraders targeting BAZ2 proteins. Such compounds were divided according to the capability to hijack POI degradation by CRBN or VHL E3 ubiquitin ligases, subdividing them into two main groups identified as series **a** and **b**, respectively. In addition, the synthesized molecules differ for the region connecting the two main PROTAC components. Notably, we opted for linkers presenting different chemical-physical features (alkyl or PEG-like), length and rigidity, in the attempt to cover all the possible combinations and maximize their capability to promote active ternary complex formation.

As anticipated, all precursors and final products were analyzed by mass spectrometry using electrospray ionization. The synthesized compounds ionized in both positive and negative modes, and MS/MS fragmentation experiments revealed a prominent fragment at m/z 95, corresponding to the loss of the neutral 5-vinyl isoxazole moiety from the POI ligand, as illustrated for compound **3a** in Figure 3.12.

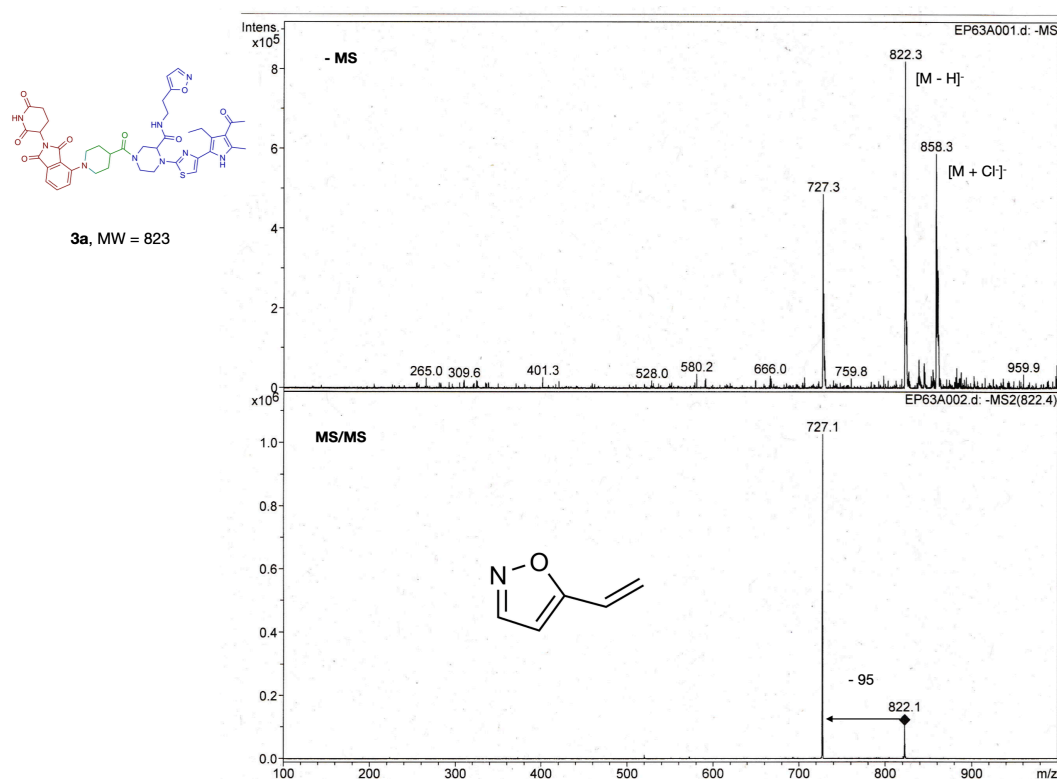


Figure 3.12: ESI-MS spectra of product **3a**: in negative ion mode (top) and by fragmentation experiment on m/z 823 $[M-H]^-$ (bottom).

The PROTAC structure is further supported by extensive NMR analysis. In detail, 1H , ^{13}C long-range bidimensional heterocorrelation were detected by HMBC experiments to elucidate the structure of final products. As exemplified for degraders **1a** and **6b**, the correlation between one proton of the piperazinyl unity and the carbon of the carbonyl group in the PEG 2 linkers, confirmed amide bond formation in the third synthetic step. This observation was proved by detecting another long-range correlation occurring between the same carbonyl group and the protons in the PEG 2 linker in the VHL-based PROTAC (**6b**). Some additional significative long-range correlations involving CRBN/VHL recruiters and the POI ligand were detected, as reported in Figures 3.13, and 3.14.

All synthesized PROTAC degraders presented a purity grade ranging from 96% to 100% and

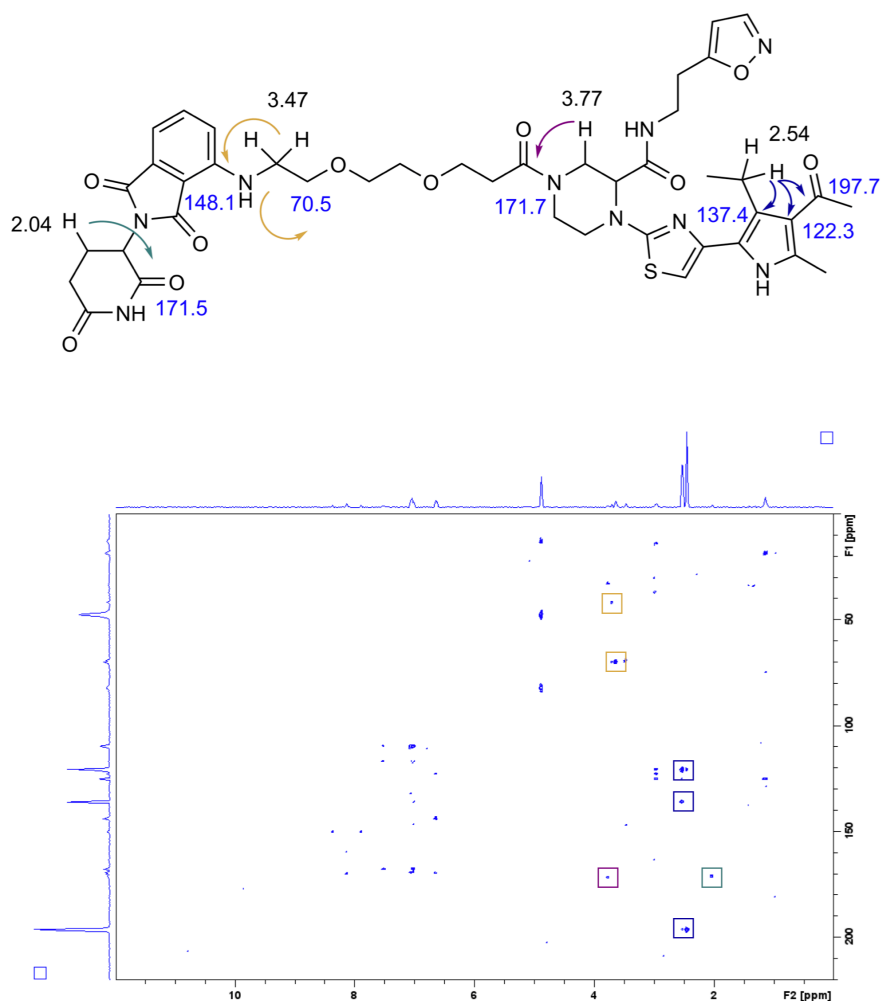


Figure 3.13: NMR spectrum of product **1a** in CD_3OD showing long range 1H , ^{13}C correlations by HMBC experiment, with the most significant correlations indicated on the molecular structure. Each correlation on the molecular structure is reported as a coloured arrow, and the corresponding signals on the spectrum presents the same colour code.

were obtained in a scale of 2-5 mg. In terms of stereochemistry, CRBN-based degraders (series **a**) presented four stereoisomers, including two enantiomers and diastereoisomers, arising from the two stereogenic centres in the thalidomide and compound **104** moieties. On the other hand, VHL-based PROTACs (series **a**) were obtained in the form of epimers, diastereoisomers differing for a single asymmetric centre in the piperazine ring of the POI ligand. Unfortunately, the HPLC conditions used to assess compound purity did not detect epimers separation, resulting in a single peak in the chromatogram.

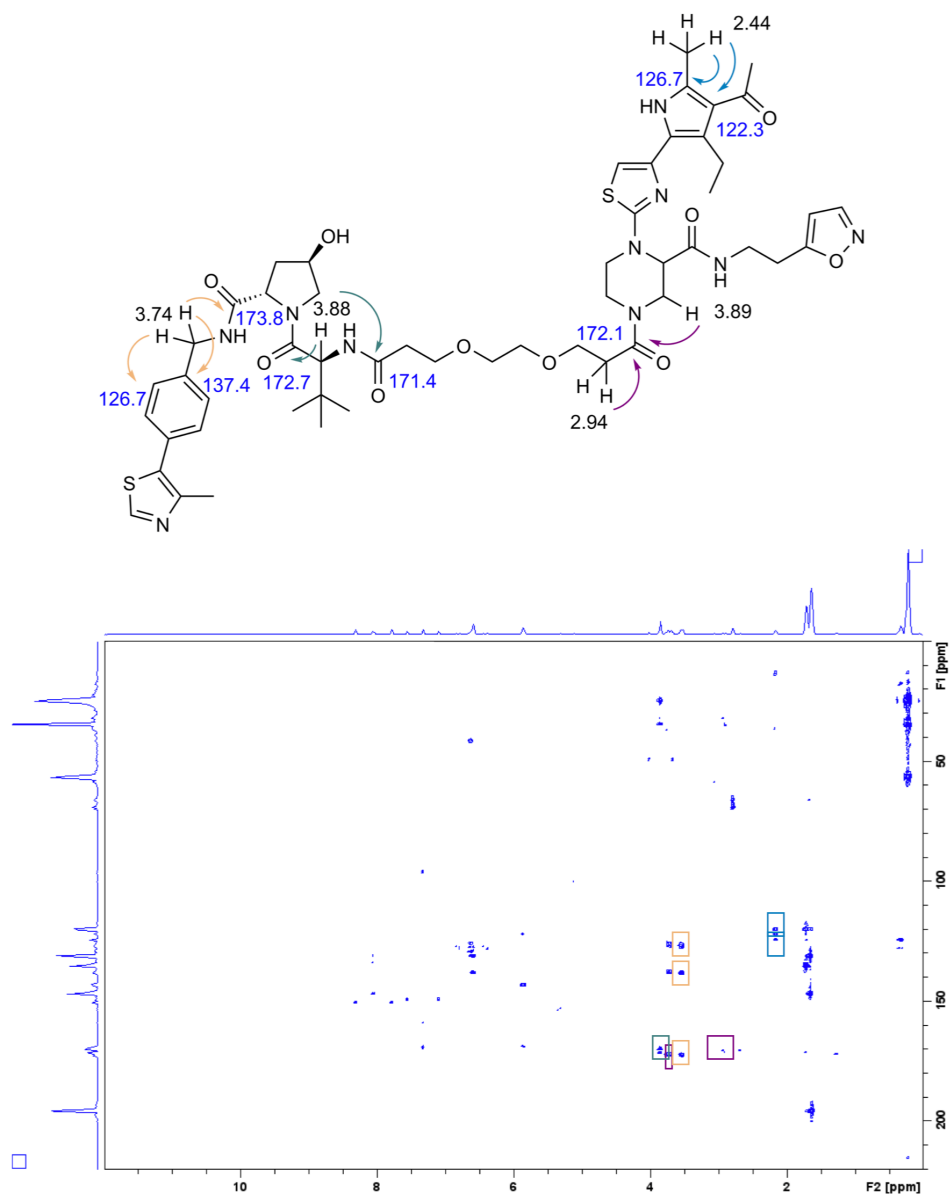


Figure 3.14: NMR spectrum of product **6b** in CD_3OD showing long range 1H , ^{13}C correlations by HMBC experiment, with the most significant correlations indicated on the molecular structure. Each correlation on the molecular structure is reported as a coloured arrow, and the corresponding signals on the spectrum presents the same colour code.

The stereoisomers separation will be pursued in future studies; however, in this work, the compounds were tested as racemic mixtures, supported by the fact that the suitable enantiomeric configuration is expected to be selected by the proteins upon ternary complex formation.

3.4 E3 Ubiquitin Ligases Production

PROTAC's ability to degrade the POI strongly depends on the formation of ternary complexes. Therefore, interactions and positioning of the target protein and E3 ligases, while engaged by PROTAC, play a fundamental role in determining the elimination of the POI. In this context, capturing a snapshot of this key event can prove the ability of the developed degraders to engage the targets *in vitro*, but can also guide additional structural modifications improving the formation of active ternary complexes.

Currently, structural characterization of ternary complexes is performed by employing either X-ray crystallographic analysis or Cryo-EM. Given our expertise in X-ray crystallography, we decided to assess ternary complex structural solution by exploiting the former technique. However, prior to exposing grown crystals to X-rays, several preparatory steps are required. First, proteins implicated in PROTAC-mediated degradation must be produced with a good purity grade. In our case, proteins involved in ternary complex formation are four: two target proteins (BAZ2A and BAZ2B bromodomains) and two E3 ligases (CRBN and VHL). While BAZ2A and BAZ2B bromodomains are routinely expressed in our laboratory, we needed to produce the E3 ligases. In the case of VHL, this protein is typically co-expressed with EloB and EloC using the *E. coli* expression system, used also for bromodomain production^[210]. In contrast, the CRBN E3 ligase is produced in insect cells through co-expression with DDB1 Δ B^[24]. Notably, CRBN and DDB1 Δ B production was performed in collaboration with the Protein Facility of Elettra Synchrotron, Trieste.

3.4.1 CRBN, DDB1 Δ B Proteins Production

CRBN E3 ligase is routinely expressed in complex with DDB1 Δ B, using the baculovirus expression system. In particular, protein production relies on insect cell infection by recombinant baculoviruses expressing the gene of interest.

For the expression of the CRBN DDB1 Δ B complex, we opted for the dsRED system, consisting of viral DNA carrying the dsRED gene partially depleted of an essential open reading frame (ORF) region and thus incapable of self-replication (bacmid). Upon homologous recombination in insect cells, CRBN and DDB1 Δ B genes are inserted in the viral DNA (bacmid), allowing the ORF restoration and viral self-replication. Successful incorporation of the target genes was confirmed by monitoring dsRED expression, indicated by a visible red colour change in the infected cell culture^[209]. Before generating recombinant viruses, viral DNA was linearized and co-transfected into Sf9 insect cells with CRBN or DDB1 Δ B DNA. Following two weeks of incubation at 27°C, the Sf9 media exhibited a red colour (Figure 3.15), confirming successful virus generation. Then, the cells were harvested by centrifugation, and the resulting supernatant (P0) was used for viral amplification. After a 5-day incubation, the cells stopped proliferating and increased in size (16.9 - 17.1 μ m) indicating that P1 virus was ready to be collected. Coloured cells were centrifuged again, and the supernatant was collected and stored for subsequent use (P1). To better identify the best growing conditions, we performed trial expression on 24 deep well plates testing: temperatures (21°C and 27°C), timing (3 or 4 days for 21°C, 2 or 3 days for 27°C) and P1 concentration (1/10, 1/100 or 1/1000). Based on the result of the first trial, large-scale CRBN-DDB1 Δ B expression was carried out by co-infecting 1 L of H5 (x4) insect cells with 5 mL of CRBN P1 and 5 mL DDB1 Δ B P1 and adding zinc acetate (50 μ M). Cultures were incubated at 27°C for 3 days.

Following incubation, cultures appeared strongly red, and cells were harvested by centrifugation. Pellets were then resuspended in lysis buffer (50 mM Tris pH 8, 500 mM NaCl, 10% glycerol, 10 mM imidazole, 2 mM DTT), while protease inhibitor mix and DNAase were also added to the mixture. After lysing cells through the ultrasonic processor, the lysate was trans-

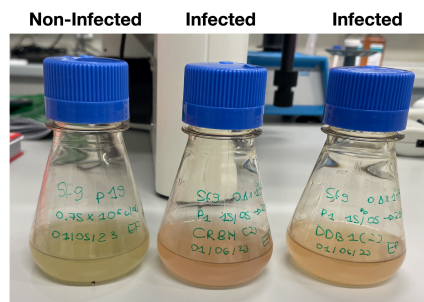


Figure 3.15: Successful homologous recombination was confirmed by DsRed expression, evidenced by a colour change in the media. The figure illustrates a comparison between Sf9 cells infected with recombinant baculovirus expressing CRBN or DDB1 Δ B and non-infected cells. The media of infected cultures displays a distinct light pink colour, in contrast to the unaltered one of the non-infected control.

ferred to appropriate tubes and centrifuged to eliminate cell debris.

The supernatant was collected and underwent the first purification step. Purification was performed as previously described [24]. Lysate was mixed with NiNTA resin, loaded on a gravity-flow column and eluted with an imidazole gradient (50 mM Tris pH 8, 500 mM NaCl, 10% glycerol, 300 mM imidazole, 2 mM DTT). CRBN-DDB1 Δ B elution was monitored by Bradford assay, and the SDS-page confirmed the complex expression. In particular, CRBN and DDB1 hold a molecular weight of 53.2 kDa and 96.3 kDa, respectively, appearing as two distinct bands on the SDS-page upon heat denaturation (Figure 3.16).

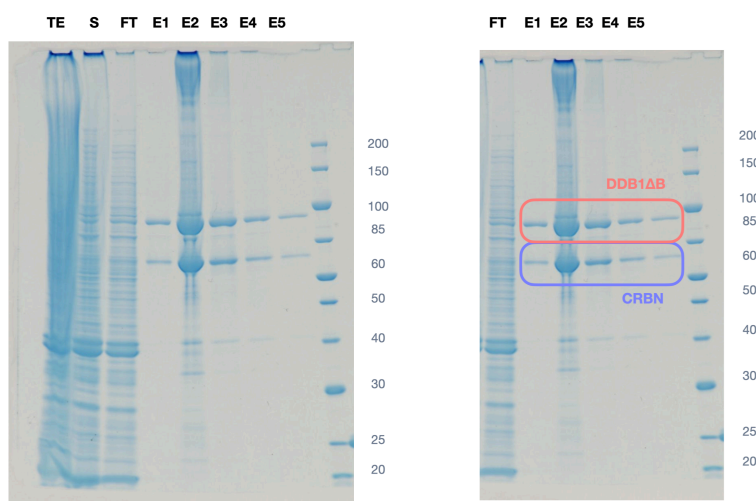


Figure 3.16: SDS-page gel of affinity chromatography. Total extract (TE), supernatant (S), flow through (FT) and eluted (E) fractions were loaded. On the right panel, CRBN and DDB1 Δ B are highlighted in blue and red, respectively.

Subsequently, proteins were loaded on HiTrap Q HP anion exchange column, previously equilibrated with buffer A (25 mM Hepes pH 7.4, 1mM TCEP). Elution was done using a linear gradient to buffer B (25 mM Hepes pH 7.4, 1 M NaCl, 1mM TCEP). CRBN-DDB1 Δ B purity grade was evaluated by SDS-page gel. Lastly, the complex was concentrated and injected onto Superdex200 16 60, eluting with appropriate buffer (50 mM HEPES pH 7.4, 200 mM NaCl, 1mM TCEP). The chromatogram exhibited a primary peak with a minor shoulder adjacent to

it. Fractions obtained from the two peaks were collected separately, and SDS-page gel revealed that both samples contained the CRBN DDB1 Δ B complex (Figure 3.18).

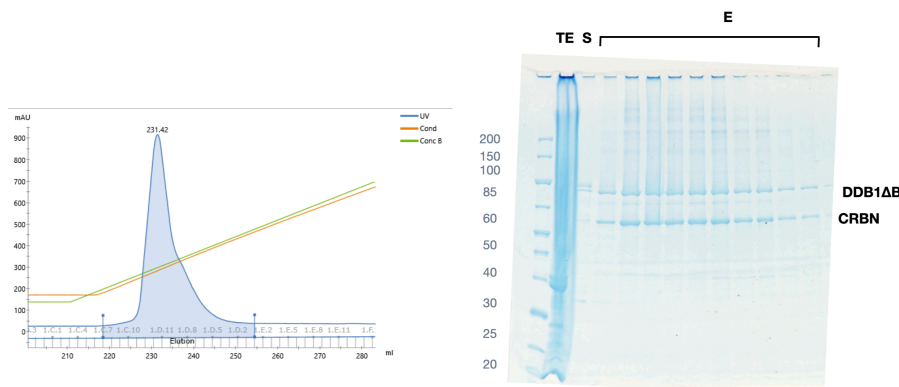


Figure 3.17: On the left is reported the anion exchange purification chromatogram, while eluted (E) fractions, total extract (TE) and supernatant (S) were loaded on SDS-page gel, as illustrated on the right.

The final purity grade in the second trial was enhanced by performing anion-exchange purification immediately after affinity chromatography, which allowed us to remove a contaminant, visible as a faint band below CRBN on the SDS-page gel (Figure 3.19). Despite this improvement, the overall purity of the CRBN-DDB1 Δ B complex remained suboptimal, and further optimization will be necessary in future trials. Nonetheless, we opted to attempt crystallization, even though the purity was not ideal.

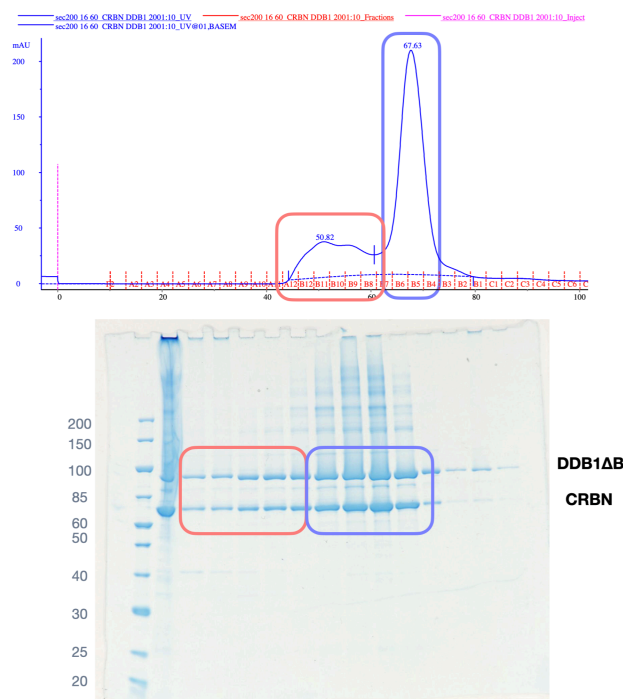
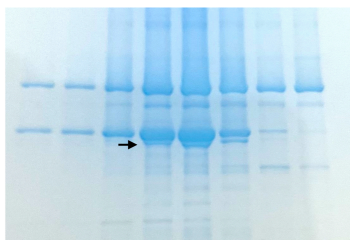


Figure 3.18: The upper panel displays the SEC chromatogram, with the two distinct peaks highlighted in different colours. The corresponding SDS-page analysis of the collected and eluted fractions from each peak is shown below, with fractions colour-coded to match their respective peaks.

SEC eluted fractions: first trial



SEC eluted fractions: second trial

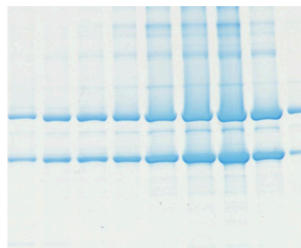


Figure 3.19: Figure illustrates SDS-pages loaded with SEC eluted fractions and compares the first (left) and the second (right) purification trials. In the latter anion exchange was performed immediately after affinity chromatography, while in the former protein was incubated overnight after purification on NiNTA resin. The first attempt reveals an additional band with a molecular weight of approximately 45 kDa, located just below the CRBN band and indicated by a small arrow.

3.4.2 VHL, EloB/C Proteins Production

VHL, EloB and EloC were expressed as described [210]. Proteins expression was performed in LB media supplemented with Ampicillin and Streptomycin, and induced by thio- β -D-galactopyranoside (IPTG) at 0.7 OD₆₀₀. The cell culture was then grown at 24°C, overnight and the following day harvested by centrifugation. Cell pellets were resuspended in lysis buffer (20 mM Tris pH 8.0, 500 mM NaCl, 10 mM imidazole, 1x complete protease inhibitor cocktail) and lysed using the ultrasonic processor. Before a second round of centrifugation to remove cell debris, the lysate was incubated for 30 minutes at room temperature with DNaseI and MgCl₂.

Lysate was filtered and loaded on a HisTrap HP column, previously equilibrated with buffer A (20 mM Tris pH 8.0, 500 mM NaCl, 10 mM imidazole). Impurities were eliminated in the flow through during the washing phase, while the His₆-tagged complex was retrieved by increasing the buffer B (20 mM Tris pH 8.0, 500 mM NaCl, 500 mM imidazole) concentration to 45%. The tag was removed by directly adding TEV protease to the protein complex and loading it onto a snakeskin. After overnight incubation, proteins were loaded again on the HisTrap HP column, and the cleaved complex was eluted in the flow through. Salt concentration was reduced by diluting the collected fractions and loading them on HiTrap, CaptoQ column. Proteins were eluted by employing a linear gradient to high salt concentration (1 M NaCl). Collected fractions were concentrated and directly injected into the Superdex75 16/60 Prep Grade column.

The collected samples and fractions were denatured and directly loaded onto SDS-page to evaluate the purity of the complex. After four purification steps, the complex exhibited a high degree of purity, with three distinct bands corresponding to VHL (24.0 kDa), EloB (13.1 kDa), and EloC (12.8 kDa) clearly visible on the gel. No additional impurities were detected.

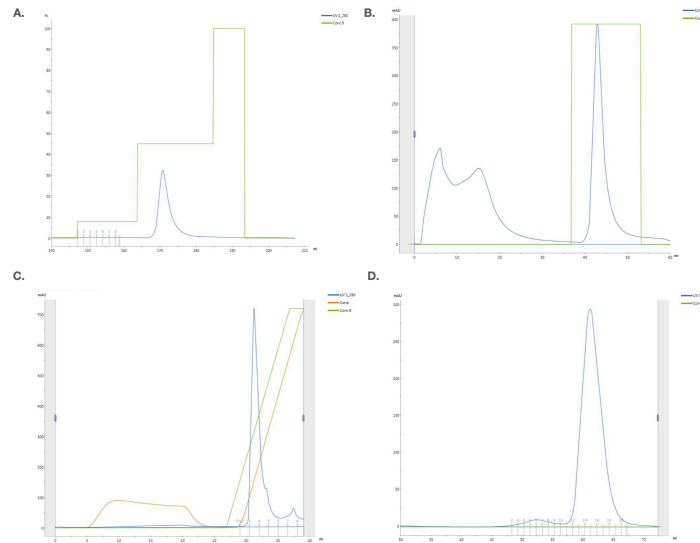


Figure 3.20: Panel **A** illustrates chromatogram of the first affinity chromatography, carried out using three imidazole concentrations: 8%, 45% and 100%. The complex was eluted in the second condition. On panel **B** is reported the chromatogram performed post-tag removal, showing the elution of the complex in the flow through while the remaining tag complex was eluted by increasing imidazole concentration. Panel **C** illustrates the third purification step chromatogram. Such a step was performed on the anion exchange column and proteins were eluted by increasing salt concentration, detected by conductivity rising (depicted in orange on the chromatogram). Last panel **D** reports the SEC chromatogram obtained with a Superdex 75 16/60 Prep Grade column, representing the final purification step.

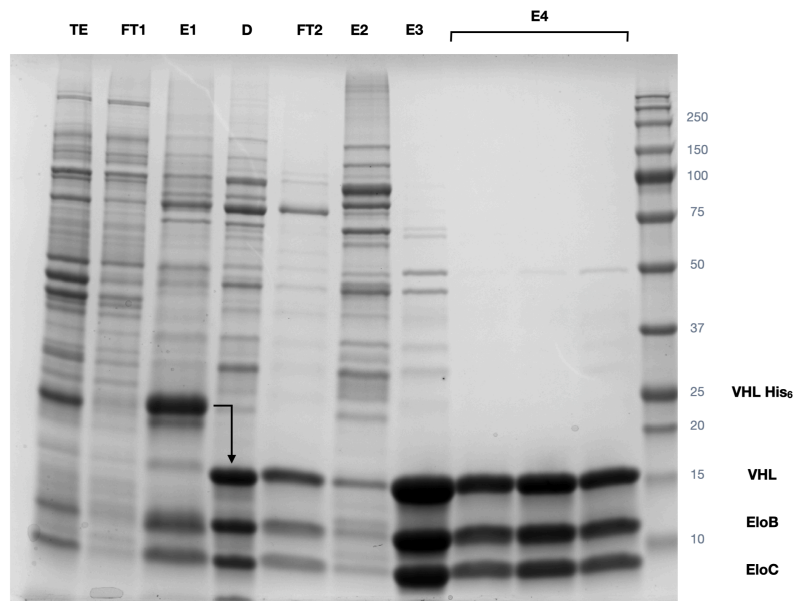


Figure 3.21: SDS-page gel with different steps of purification. From left to right: TE (total extract), FT1 (flow through of the first affinity), E1 (eluted fractions of the first affinity chromatography), D (dialyzed sample) (small arrow indicating the same sample before and after His₆ tag cleavage), FT2 (flow through of the second affinity), E2 (eluted fractions of the second affinity chromatography), E3 (eluted fractions of anion exchange chromatography), E4 (eluted fractions of SEC) were loaded.

3.5 Crystallization of PROTAC-Induced Ternary Complexes

Once the degraders and proteins are produced, structural elucidation via X-ray crystallography requires the formation of protein crystals. The latter are highly ordered, three-dimensional arrangements of protein molecules obtained by driving the proteins in solution to a supersaturated state under controlled conditions. In our case, we aimed for crystals containing ternary complexes assembling inside the cells and driving POI degradation. In other words, crystals should contain three main components: E3 ubiquitin ligase (CRBN or VHL), PROTAC degraders (belonging either to series **a** or **b**, depending on the targeted E3) and POI (BAZ2A or BAZ2B bromodomains).

3.5.1 CRBN-based Ternary Complexes

As a first approach to the crystallization of ternary complexes induced by PROTAC, we decided to investigate and get inspiration from released structures on the protein data bank (PDB). However, the amount of ternary complexes structures characterized by X-ray crystallography is limited, and only a few examples are reported in the literature^[24]. For CRBN-based ternary complexes, the principal article involves the structural characterization of BRD4 PROTACs. Therefore, we resemble similar protein/compound ratios employed to obtain such crystals. In particular, we mixed CRBN-DDB1 Δ B (E3 ligase) and BAZ2A or BAZ2B bromodomains stoichiometrically (50 μ M) at similar concentrations to the one reported in ^[24]. While BRD4-based PROTACs are employed as a racemic mixture composed of two enantiomeric forms, our degraders hold two asymmetric centres, giving rise to four different stereoisomers. Consequently, we opted to add our degraders at a concentration four times that of the proteins (200 μ M). This approach considers that only one of four degrader molecules is in the active conformation and capable of simultaneously engaging both target proteins.

Once we established proteins-degrader ratios, we needed to test various crystallization conditions. In particular, we exploited the following available screenings: Morpheus, Proplex, JCSG, and SG1. After a few weeks of incubation at 4°C or 20°C, most conditions presented mucilage-like precipitations, while successful crystal formation was observed only in the SG1 screen. Two different crystal forms were observed: tiny with a needle-like shape and plane-squared crystals (Figure 3.22). The latter exhibits no birefringence under polarized light, a feature typically associated with crystal formation. During collection, they were identified as non-crystalline, supporting the initial hypothesis. To confirm the conditions' reproducibility and detect even better crystallization mixes, we performed additional screenings by slightly changing pH, PEG and salt concentrations. Within a few weeks, the first type of crystal shape grew similarly to the previous one. Crystals were obtained in all the different ternary complex combinations, including either POIs (BAZ2A and BAZ2B) and three synthesized CRBN-based PROTAC degraders.

3.5.2 VHL-based Ternary Complexes

Similarly to CRBN-based PROTACs, we exploited ternary complex structures present in the literature. We opted for a final concentration of 160 μ M concerning BAZ and VHL proteins. Although most ternary complexes reported 1 : 1 : 1 stoichiometric ratio between the different components, we preferred to increase PROTAC concentration moderately (240 μ M)^[23]. Given that PROTACs were obtained as a racemic mixture holding two different enantiomeric forms, we preferred slightly improving compound concentration to reduce the probability of generating inactive binary complexes. For crystallization, we opted for SG1 screening, which resembled more conditions reported in the literature.

Within a week, crystals formed and presented cubical or plane-squared shapes. By visual

inspection under the microscope, most of the crystals were not single but assemblies of smaller crystals growing simultaneously (Figure 3.23). Successful crystal formation was also achieved by slightly varying pH, PEG concentration, and salt levels. Notably, crystals grew mainly in combination with BAZ2A bromodomain, while BAZ2B ternary complexes led to crystal formation only in one case. Most of the crystals originated from the BAZ2A bromodomain in complex with degraders (**6b**, **7b**) featuring PEG-like linkers, and they successfully grew at 20°C. In contrast, the only BAZ2B-derived crystals formed under lower temperature conditions (4°C) in the presence of the **3b** PROTAC, which contains an aliphatic-type linker.

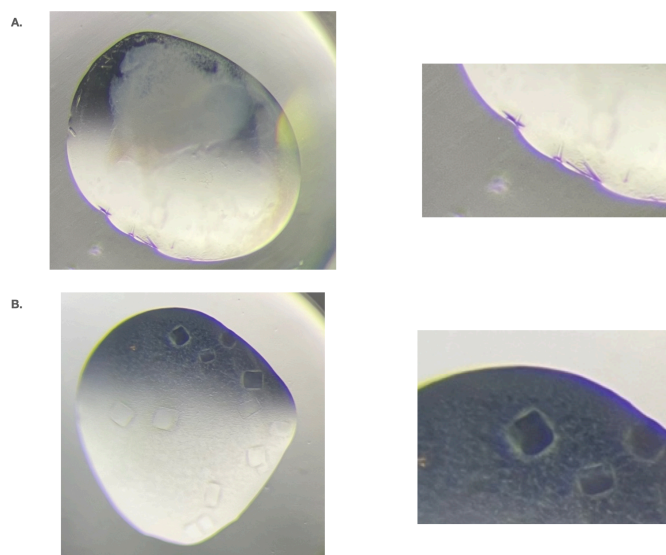


Figure 3.22: Panel **A** reports the first type of crystals formed using SG1 screening. Crystals present a needle-like shape, visible also in the magnification alongside. On panel **B** is reported the second type of crystal formation and the related magnification alongside.

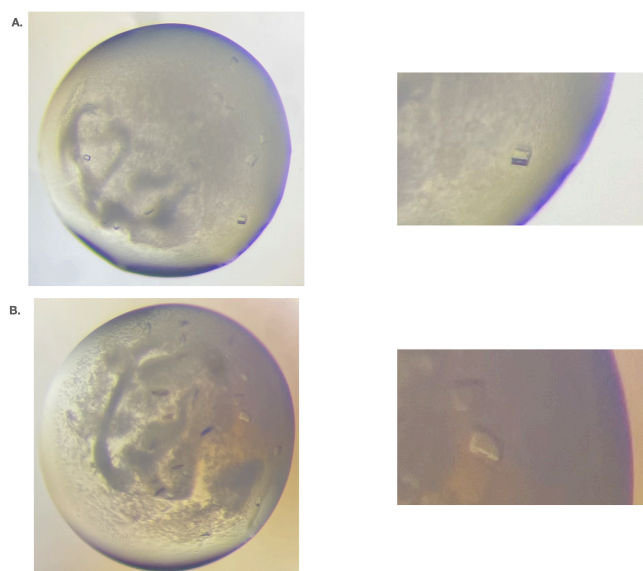


Figure 3.23: Panel **A** shows the results of SG1 screening, highlighting the first type of crystal formation with a cubic shape. Panel **B** presents the second type of crystal, exhibiting a planar morphology.

3.6 Structure Solution

Obtained crystals were exposed to X-ray at synchrotron sources and CRBN and VHL structures were determined as described in the Section 4.6.

3.6.1 CRBN-based Ternary Complexes

Upon X-ray diffraction analysis, all CRBN-derived crystals were found to belong to the orthorhombic crystal system, with a space group designation of P 21 21 21, with one molecule per asymmetric unit.

Unfortunately, the structure solution revealed that the crystals were composed solely of the DDB1 Δ B protein (Figure 3.24). Comparing the obtained structure with the available ternary complex of CRBN-DDB1 Δ B (PDB 6BOY), we identified several differences. Notably, the released ternary complex structure appeared less compact than the one we determined from our crystals. This observation was confirmed through root mean square deviation (RMSD) analyses (using Chimera), which showed a significant difference in the β -propeller C region (highlighted in green in both the sequence and structure). As shown in Figure 3.25, the released DDB1 Δ B structure (light blue) exhibits a greater distance between the A and C β -propellers, while our DDB1 Δ B structure (pink) adopts a more compact conformation. Such difference should be ascribed to the presence of CRBN protein (yellow in the Figure 3.25), normally accommodated in between the two regions. Other less relevant differences were detected in the loop region, determined by a different crystal packing. We also compared our results with available DDB1 structures (PDB 9BBE) (blue) and noticed a more similar conformation compared to the one in complex with CRBN. RMSD analysis revealed that the main differences were localized in the loop regions, likely due to variations in crystal packing, while excluding the additional β -propeller observed in the DDB1 structure (Figure 3.26).

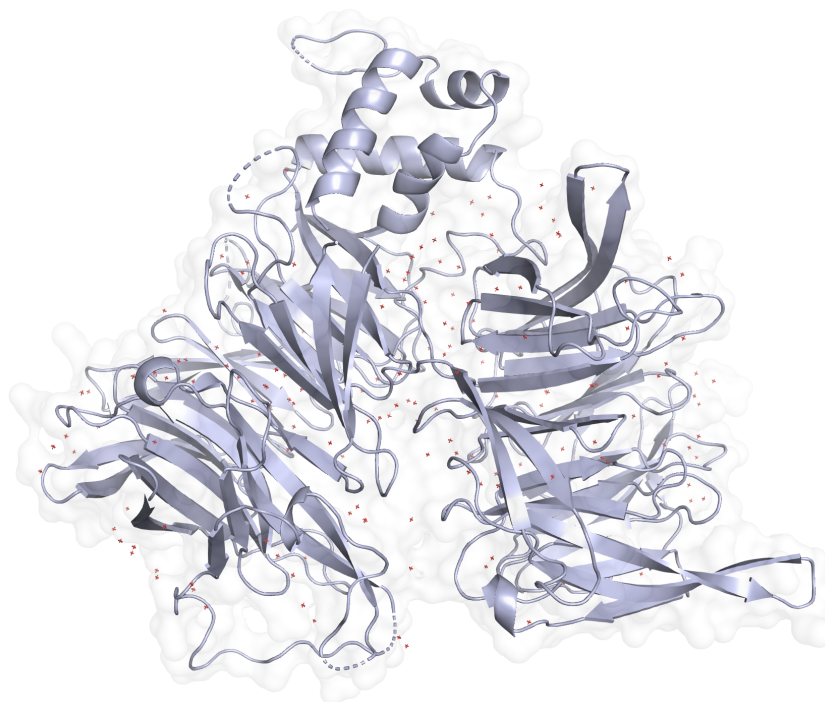


Figure 3.24: Ribbon diagram of determined DDB1 Δ B structure.

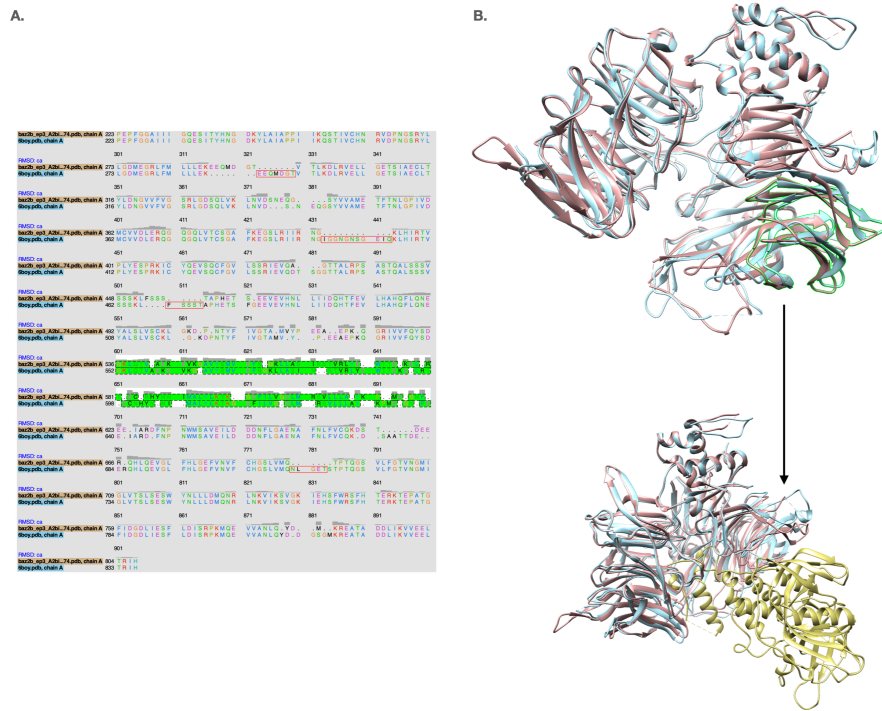
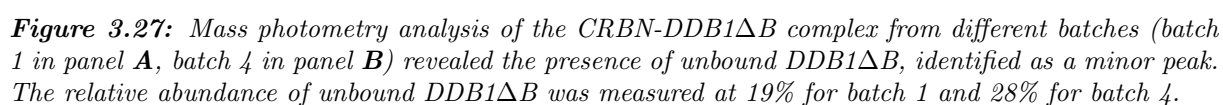
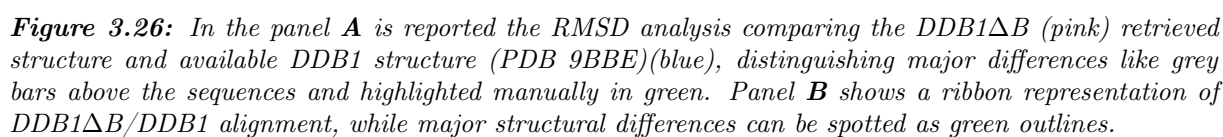


Figure 3.25: Panel **A** reports the result of RMSD analysis comparing our DDB1ΔB structure with the released one belonging to a CRBN-based ternary complex (PDB 6BOY). RMSD differences are represented as grey bars above the sequence comparison. Major differences are highlighted manually in green. On panel **B** are shown two ribbon representations of the DDB1ΔB structure (in pink our DDB1ΔB structure and in light blue the released one), aligned according to their structural conformation. Similar to the RMSD analysis on panel **A**, major structural differences are highlighted like a green outline. Below is also reported the CRBN structure (yellow) in complex with DDB1ΔB, showing the region normally occupied by the protein while bound to DDB1ΔB.

Lastly, we investigated putative reasons behind the crystallization of only DDB1ΔB proteins, hypothesizing an increased production of DDB1ΔB during co-expression and purification with CRBN. To confirm that, we analyzed the produced complex using a mass photometer, a technology detecting the exact mass of biomolecules in solution without labelling it. The results confirmed the presence of the CRBN-DDB1ΔB complex but also revealed an excess of unbound DDB1ΔB. Given that both proteins were tagged with His₆, we speculated that during the initial affinity chromatography step, the CRBN-DDB1ΔB complex and an excess of unbound DDB1 were eluted together. In the final purification step, size-exclusion chromatography (SEC) was employed, however, the last collected fractions presented an excess of unbound DDB1ΔB that unfortunately was not detected by the SEC chromatogram or SDS-page gel. Subsequently, only the mass photometer analysis successfully discriminated between this excess of DDB1ΔB and the complex, as reported in Figure 3.27.

Table 3.1: Data Collection and Refinement Statistics for DDB1ΔB, obtained by mixing CRBN-DDB1ΔB, **3a** and BAZ2B bromodomain.

	DDB1ΔB
Beamline	ESRF ID30-A3
Space group	P 21 21 21
Unit-Cell parameters (Å)	a = 67.94 b = 117.78 c = 118.26
Resolution (Å)	83.45-2.49 (2.40-2.40)
R _{merge} (%)	0.110 (0.869)
R _{meas} (%)	0.119 (0.966)
R _{pim} (%)	0.045 (0.358)
Number of molecules per asymmetric unit	1
Total number of observations	256854 (27619)
Total number unique	37241 (3883)
Mean ((I/sd(I)))	10.9 (2.2)
CC ^{1/2}	0.997 (0.781)
Completeness	98.5 (99.4)
Multiplicity	6.9 (7.1)
R _{work} /R _{free} (%)	20.5/26.1
Bond lengths (Å)	0.008
Bond angles (°)	1.004



3.6.2 VHL-based Ternary Complexes

X-ray diffraction analysis of crystals derived from VHL-based ternary complexes revealed that the crystals were composed of VHL EloB/C proteins bound to two distinct PROTACs. Binary complexes, resulting from mixtures of BAZ2A Bromodomain, VHL EloB/C, and the PROTACs **6b** and **7b** (which contained PEG 2 and PEG 3 linkers, respectively), were successfully determined. However, the PROTACs electron density map was retrieved only until the first ether group in the PEG linker, while the remaining PROTACs components were not visible (Figure 3.29).

Crystals composed of VHL EloB/C and compound **7b** exhibited two distinct crystal packing arrangements, corresponding to the space groups $P 4_1 2 2$ and $C 2 2 21$. The space group $P 4_1 2 2$, which belongs to the tetragonal crystal system, displayed a crystal packing where four protein monomers were arranged in an almost identical conformation (Figure 3.28, A.). In contrast, the $C 2 2 21$ space group exhibited a crystal packing where two VHL EloB/C monomers were arranged specularly (Figure 3.28, B.). The $P 4_1 2 2$ crystals have four molecule per asymmetric unit, while the $C 2 2 21$ crystal has two. The observed packing motifs were consistent with those reported in previously published structures of VHL EloB/C proteins, either in complex with other ligands or in their apo conformation (e.g., PDB 3ZRC, PDB 8VL9).

The structure obtained from the mixture of BAZ2A, VHL EloB/C, and compound **6b** showed identical packing to the $P 4_1 2 2$ space group, with four VHL EloB/C monomers arranged in a repeating pattern (Figure 3.28). Similarly, the last retrieved structure of VHL EloB/C in complex with PROTACs showed the same conformation of four monomers with a $P 4_1 2 2$ space group designation; however, no electron density was detected in the ligand-binding pocket (Figure 3.30). The reasons behind the lack of **3b** compound in the VHL pocket could be possibly ascribed to different crystallization conditions, including different temperatures (4°C instead of 20°C) or BAZ2A bromodomain substitution with BAZ2B. Notably, the temperature or BAZ2B bromodomain addition could have altered the binding environment within the VHL protein, resulting in the lack of an electron density map in the pocket. However, given the numerous variables that influence the crystallization process, it is challenging to determine a single discriminating factor responsible for the absence of compound **3b** in the VHL pocket.

Unfortunately, crystallization experiments did not lead to a ternary complex structure solution, however, they can give insights about possible new trial conditions. Specifically, adjustments to key parameters, such as the protein-to-ligand ratio or temperature, may increase the likelihood of obtaining VHL-based ternary complex crystals in subsequent trials.

Table 3.2: Data Collection and Refinement Statistics for VHL-EloB/C structure, obtained by mixing VHL-EloB/C, **3b** and BAZ2B bromodomain or **6b** and BAZ2A bromodomain.

	VHL-(3b-BAZ2B)	VHL-6b-(BAZ2A)
Beamline	Elettra XRD2	Elettra XRD2
Space group	P 4 ₁ 2 2	P 4 ₁ 2 2
Unit-Cell parameters (Å)	a = 93.45 b = 93.45 c = 360.24	a = 93.09 b = 93.09 c = 358.36
Resolution (Å)	360.24-3.29 (3.10-3.10)	93.09-2.80 (2.70-2.70)
R _{merge} (%)	0.454 (2.441)	0.204 (2.275)
R _{meas} (%)	0.463 (2.488)	0.209 (2.321)
R _{pim} (%)	0.093 (0.481)	0.043 (0.457)
Molecules per asymmetric unit	4	4
Total number of observations	724465 (126066)	1036266 (117445)
Total number unique	29158 (4758)	43752 (4611)
Mean ((I/sd(I)))	7.1 (1.7)	11.3 (1.7)
CC ^{1/2}	0.995 (0.764)	0.997 (0.773)
Completeness	96.4 (100.0)	98.0 (100.0)
Multiplicity	24.8 (26.5)	23.7 (25.5)
R _{work} /R _{free} (%)	25.8/32.7	23.0/28.6
Bond lengths (Å)	0.012	0.009
Bond angles (°)	1.34	1.19

Table 3.3: Data Collection and Refinement Statistics for VHL-EloB/C structure, obtained by mixing VHL-EloB/C, **7b** and BAZ2A bromodomain.

	VHL-7b-(BAZ2A)	VHL-7b-(BAZ2A)
Beamline	Elettra XRD2	Elettra XRD2
Space group	P 4 ₁ 2 2	C 2 2 21
Unit-Cell parameters (Å)	a = 93.74 b = 93.74 c = 357.20	a = 62.59 b = 69.65 c = 359.80
Resolution (Å)	89.30-2.80 (2.70-2.70)	89.95-2.88 (2.75-2.75)
R _{merge} (%)	0.200 (2.248)	0.130 (1.583)
R _{meas} (%)	0.204 (2.295)	0.136 (1.658)
R _{pim} (%)	0.041 (0.459)	0.039 (0.486)
Molecules per asymmetric unit	4	2
Total number of observations	1078954 (113571)	241193 (30919)
Total number unique	44079 (4634)	20494 (2738)
Mean ((I/sd(I)))	13.7 (1.8)	12.9 (1.6)
CC ^{1/2}	0.998 (0.615)	0.998 (0.631)
Completeness	97.7 (100.0)	97.1 (100.0)
Multiplicity	24.5 (24.5)	11.8 (11.3)
R _{work} /R _{free} (%)	21.1/25.5	23.5/29.4
Bond lengths (Å)	0.009	0.010
Bond angles (°)	1.15	1.21

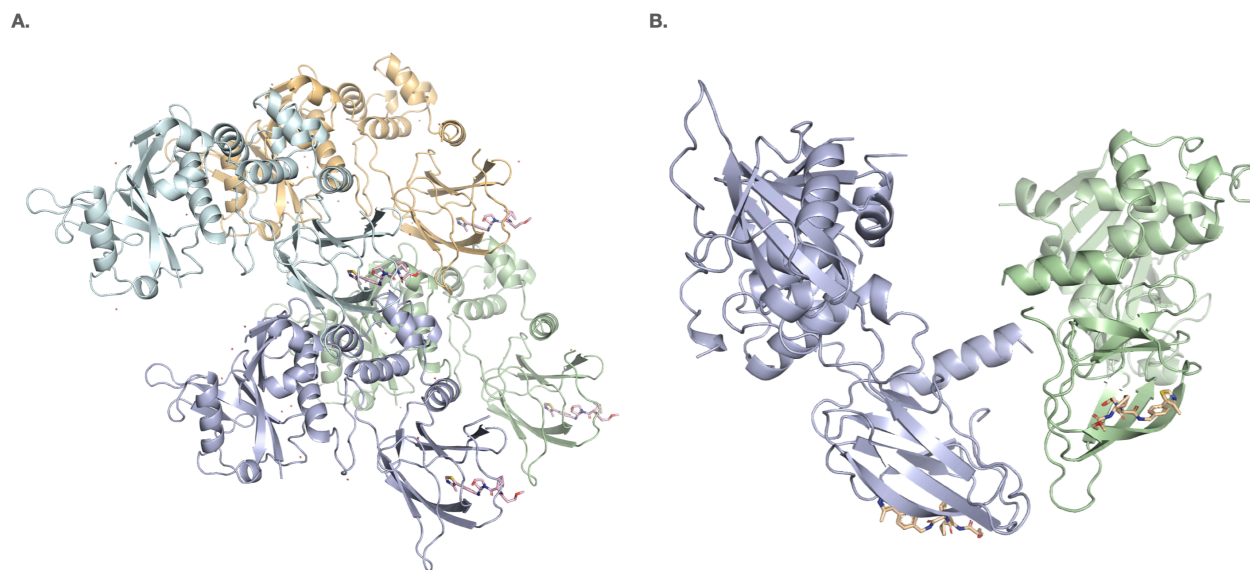


Figure 3.28: Panel A and B report a ribbon diagram of VHL EloB/C bound to compounds **6b** and **7b**, respectively. The comparison highlights the different crystal packing between the two determined structures. In addition, VHL EloB/C monomers are depicted in different colours.

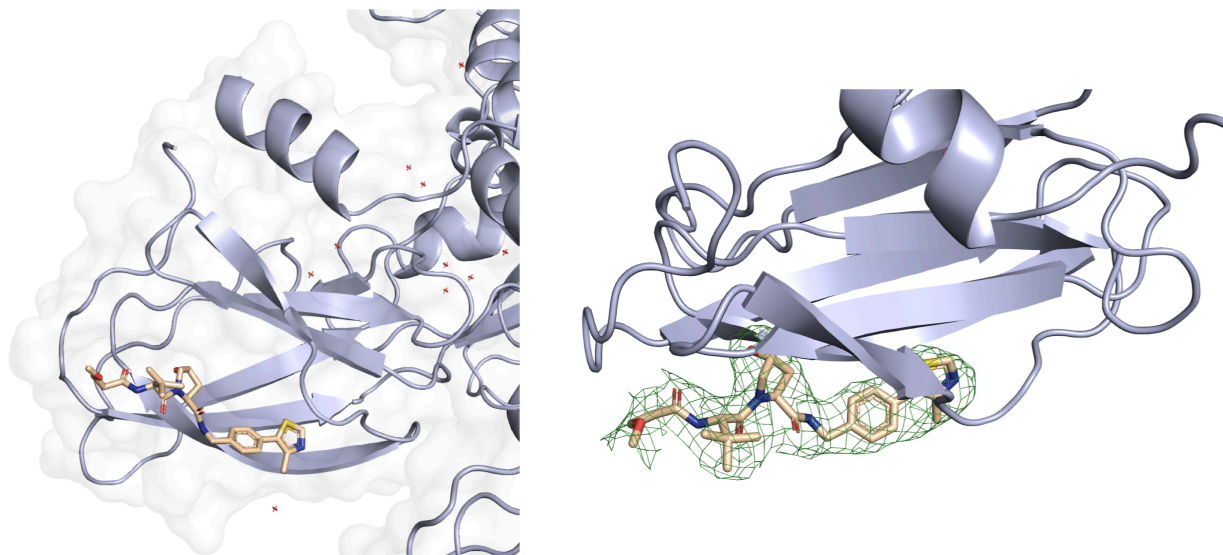


Figure 3.29: Ribbon representation of VHL EloB/C in complex with PROTAC **7b**, alongside is reported the corresponding polder OMIT map in green with contour level at 3.0σ .

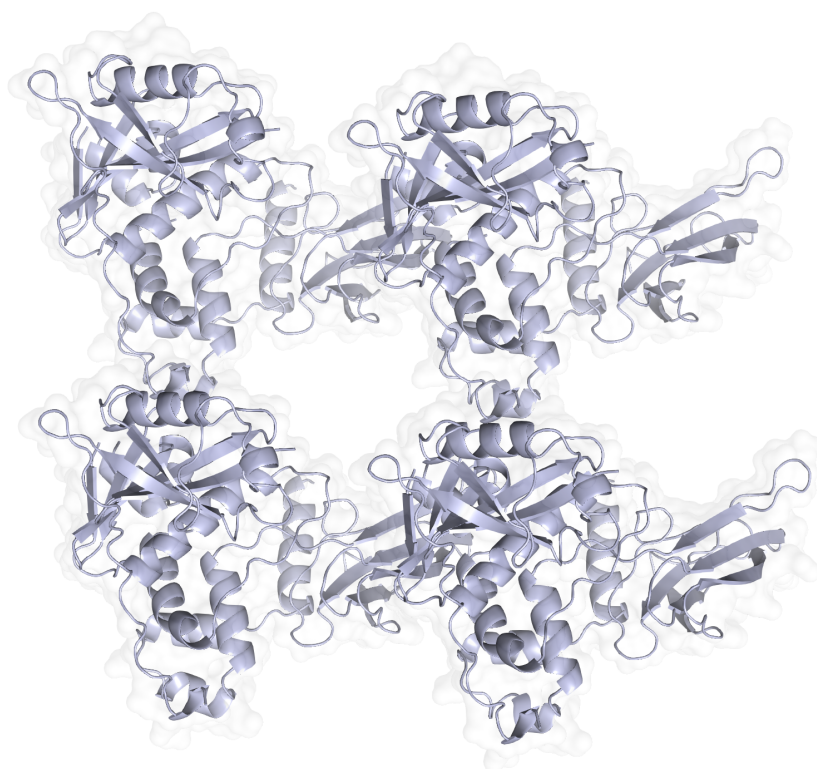


Figure 3.30: Ribbon diagram of VHL EloB/C obtained by mixing the complex with BAZ2B bromodomain and compound **3b** at 4° C.

3.7 Cell-based Activity of Developed Degraders

Developed degraders also needed to be tested for their activity in appropriate cell lines. We investigated their capability to degrade our target protein by testing a single concentration and two different time points.

The first tested compounds belong to series **a**, thus recruiting CRBN E3 ligase through pomalidomide moiety. We opted for a high concentration that allowed us to discriminate between active and non-active compounds. PROTACs were tested on PC3 cancer cell lines treated with a final concentration of 50 μ M. Protein degradation was measured by western blot analysis and detected at 4 hours and 6 hours upon the treatment. Unfortunately, none of the developed compounds was actively degrading the BAZ2A protein.

Based on the outcome of this first experiment, we decided to change the conditions moderately. Notably, we tested VHL-based PROTACs (series **b**) at a lower concentration (20 μ M) and extended the time course of the treatment. PC3 cancer cells were treated with three VHL-based PROTACs (**1b**, **2b**, **3b**), and degradation was detected using western blot analysis at 6 and 24 hours. Differently from series **a**, compounds **1b** and **3b** were actively degrading BAZ2A proteins. In particular, **1b** molecule maintained degradation until 24 hours, while **3b** PROTAC showed reduced BAZ2A concentration only after 6 hours of treatment. However, considerable variability observed at the 24-hour time point undermines the consistency of **3b**'s activity in modulating BAZ2A expression at this time.

Collectively, preliminary experiments indicate that VHL-based PROTACs featuring longer linkers induced BAZ2A elimination, while no degradation activity was detected upon CRBN-based degraders. Future investigations are requested to confirm the observed results and demonstrate VHL-based PROTACs' ability to mediate BAZ2A degradation.

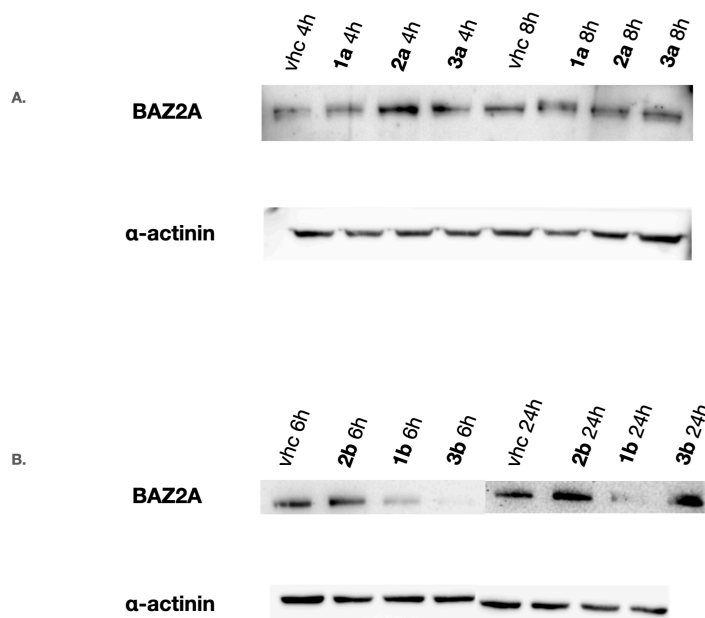


Figure 3.31: Panel **A** reports western blot analysis of PC3 cells treated with CRBN-based PROTACs (**1a**, **2a**, **3a**). Similarly, degradation mediated by VHL-based PROTACs (**1b**, **2b**, **3b**) was monitored by western blotting, as illustrated in panel **B**. A loading control (α -actinin) was used to show that the same amount of protein lysate was loaded for the two conditions. DMSO (0.1%) was used as vehicle (vhc).

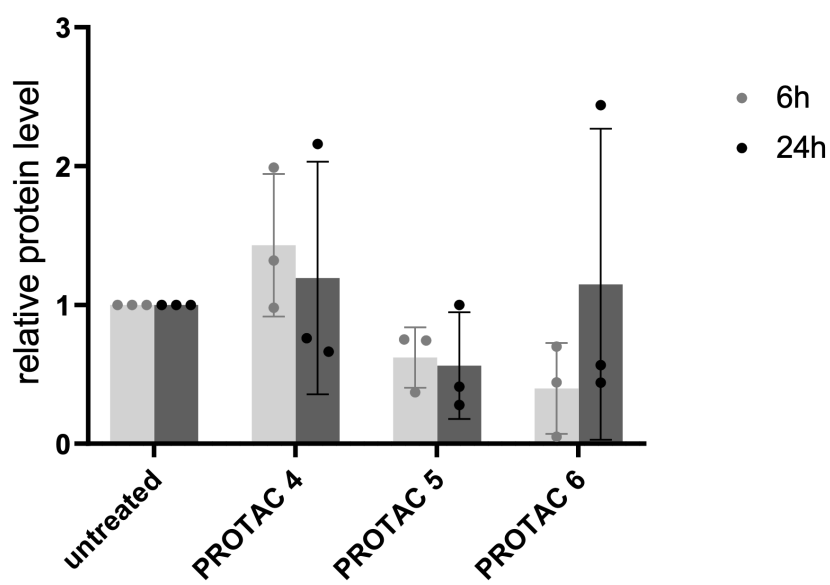


Figure 3.32: Bar charts reporting BAZ2A quantification upon **1b**, **2b** and **3b** PROTACs treatment at 6 and 24 hours. Error bars represent standard deviation (SD).

Chapter 4

Materials and Methods

4.1 Cell-based Assays

Proliferation assay

PC3 and 22Rv1 cells were cultured in RPMI 1640 medium containing 10% FBS, 1% penicillin-streptomycin and 2 mM L-glutamine. All cells were regularly tested for mycoplasma contamination.

Cells were counted with a hemocytometer and seeded in triplicates in 96-well plates at the final density of 4.5×10^3 cells/well for 22Rv1 and 2.0×10^3 for PC3. Cells were incubated overnight at 37°C, 5% CO₂, to ensure complete adhesion. The following day, the medium was replaced with RPMI 1640 containing either the carrier (0.1% DMSO) or the inhibitors (0.5 – 5.0 – 10.0 – 25.0 μ M BAZ2-ICR; 0.5 – 2.5 – 5.0 – 10.0 – 25.0 μ M GSK2801; 0.1 – 0.25 – 0.5 – 1.0 – 2.5 – 5.0 – 10.0 – 20.0 μ M). The cells were incubated for 1, 3, 6 (PC3) and 9 days (22Rv1), replacing the medium containing the carrier or inhibitors every 3 days.

Cell proliferation and viability were measured on days 1, 3, 6 and 9 by adding 10% resazurin sodium salt solution (0.03 mg/ml powder from Sigma-Aldrich) directly into each well. After 4 hours of incubation at 37°C, 5% CO₂, the absorbance at 570/600 nm was detected using a Varioskan plate reader. Cell viability was determined by measuring the reduction of the resazurin sodium salt, calculated using the formula: $(\text{Abs } x - \text{Abs } 0.1\% \text{DMSO day1}) / (\text{Abs } 0.1\% \text{DMSO day9} - \text{Abs } 0.1\% \text{DMSO day1})$. The growth rate was analyzed using the linear regression algorithm of GraphPad Prism. The model appropriateness was evaluated using the R square values and the Runs test.

siRNA Transfection

PC3 and 22Rv1 cells were plated on 6 well or 96 well plates for RNA extraction or growth curve, respectively. Cells were seeded at the final concentration of 6.0×10^4 or 4.5×10^3 for PC3 and 2.0×10^4 or 4.5×10^3 for 22Rv1 and let adhere overnight at 37°C, 5% CO₂. Transfection was performed using 50 nM esiRNA BAZ2A, esiRNA RLUC (negative control), or esiRNA KIF11 (positive control) and JetPRIME[®] reagent, as reported in the manufacturer's instructions. Cells were retreated using the same amount of esiRNA BAZ2A after 3 days and collected at 6 days for gene expression analysis. Viability was detected using 10% resazurin sodium salt solution (0.03 mg/mL powder from Sigma-Aldrich) and measured by Varioskan plate reader (absorbance at 570/600 nm).

RT-qPCR

The total RNA content was extracted from cells treated for 6 days with 25 μ M BAZ2-ICR or with the carrier and from cells treated with 50 nM siRNA against BAZA (MISSION[®] esiRNA, Merck). The RNA was extracted using the phenol-chloroform method and its concentration was measured by Nano spectrophotometer. 1 μ g of RNA per sample was retrotranscribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. The obtained cDNA was diluted 1:8 and used for RT-qPCR, performed on a Biorad CFX96 thermocycler using the Excel-Taq FAST qPCR SybrGreen (Smobio) reagent, according to the manufacturer's instructions. Every reaction was performed in triplicate and accompanied by the two negative controls (water or non-retrotranscribed RNA as template). Genes expression was normalized using the Pfaffl method with three different housekeeping genes.

Cell Cultures and Treatments

PC3 cells were cultured in RPMI 1640 medium containing 10% FBS, 1% penicillin-streptomycin and 2 mM L-glutamine. All cells were regularly tested for mycoplasma contamination.

For CRBN-based PROTACs (**1a**, **2a**, **3a**), cells were plated at the final density of 75×10^4 cells/well on a 6-well plate. Incubation at 37°C, 5% CO₂, allowed appropriate adhesion. The following day, the medium was replaced with either vehicle (0.1% DMSO) or 50 μ M **1a**, **2a** or **3a**.

Upon 4 hours or 6 hours of treatment, pellets were collected and stored at -80°C.

For VHL-based PROTACs (**1b**, **2b**, **3b**), cells were seeded at the final density of 70×10^4 in the 6 hours treatment or 45×10^4 in the 24 hours treatment on 6-well plates. Cells were incubated at 37°C, 5% CO₂, to ensure complete adhesion. The following day, cells were treated by replacing the medium either with the carrier (0.1% DMSO) or 20 μ M **1b**, **2b** or **3b**.

Upon 6 hours or 24 hours of treatment, pellets were collected and stored at -80°C.

Western Blot

Cells were lysed using RIPA buffer and protein concentration was determined by Bradford Reagent (Sigma-Aldrich), according to the manufacturer's instructions. 25 μ g of proteins per sample in Laemmli Buffer 4x were run in a 6% or 8% polyacrylamide gel until complete separation. The proteins were then blotted on a PDVF (polyvinylidene difluoride) membrane, which was blocked in 5% skimmed milk and 0.1% Tween 20 in TBS for 2h at room temperature and then treated with the appropriate primary (1:1000 anti-BAZ2A Abcam ab290639 or 1:4000 anti- α -actinin Santa Cruz Biotechnology sc-17829; 4°C overnight) and secondary (1:10 000 goat anti-mouse HRP Invitrogen 62-6520 or 1:10 000 goat anti-rabbit HRP Cohesion Biosciences CSA2115; 2h at room temperature) antibodies and revealed by chemiluminescence (ECL Bio-Rad).

4.2 Synthesis and Characterization of BAZ2A/B PROTACs

Chemicals and Reagents

All chemicals and reagents were purchased from ChemSpace, BLDPharma, Sigma Aldrich, Fluorochem Ltd, or Avantor-VWR with a final purity of at least 95%. Unless otherwise specified, all reagents were used as received without further purification.

Purification Methods

All final products and precursors were purified using either preparative thin-layer chromatography (PLC) or column chromatography, as described below:

- Final products or precursors were loaded on 20 x 20 cm Merck Kieselgel 60 F254 plates with a layer thickness of 0.5 mm and eluted with appropriate conditions. Bands were carefully scraped, and compounds were recovered with dichloromethane/ethanol 8:2.
- Final products or precursors were loaded on silica column packed with NORMASIL 60[®] silica gel (particle size: 40–63 μm). Elution was performed by using appropriate isocratic conditions. Collected fractions were analyzed by thin-layer chromatography under UV light.

Structural Characterization

The structural characterization of either final products or precursors was performed using the following techniques:

- Nuclear Magnetic Resonance (NMR) Spectroscopy: spectra were acquired on a Bruker Avance 400 MHz spectrometer. For proton ^1H NMR and long-range heteronuclear ^1H , ^{13}C correlations (HMBC), a 5 mm BBI probe was used at 400 MHz. For carbon ^{13}C NMR, a BBO probe was employed at 100 MHz. The samples were dissolved in either deuterated chloroform (CDCl_3) or deuterated methanol (CD_3OD). Chemical shifts were referenced to the solvent residual signals: ^1H NMR in CDCl_3 (7.27 ppm) and ^{13}C NMR (77.00 ppm); or ^1H NMR in CD_3OD (3.31 ppm) and ^{13}C NMR (49.00 ppm). Coupling constants (J values) were reported in hertz (Hz). NMR spectra analysis was performed using Topspin Software (Bruker).
- Mass Spectrometry (MS): spectra were recorded on a Bruker Esquire-LC spectrometer. Samples in methanol solution were introduced via direct infusion using electrospray ionization (ESI) for analysis detecting signals in positive and/or negative ion mode.
- High-Resolution Mass Spectrometry (HRMS): High-resolution mass spectrometry was performed using an Orbitrap Fusion Tribrid mass spectrometer.
- Optical rotation measurements were performed using a JASCO DIP-181 digital polarimeter. The instrument was calibrated prior to use with a standard solution and set to wavelengths $\lambda = 589\text{ nm}$ (Na line), $\lambda = 577\text{ nm}$ (Hg line) for all measurements.

Data Analysis

Products and precursors were visualized using thin-layer chromatography (TLC). Analyses were performed on Merck Kieselgel 60 PF254 plates. The plates were developed with appropriate solvent systems, and the separated compounds were observed under UV light to assess their purity and confirm the presence of the desired products.

The purity of the final products was assessed high-performance liquid chromatography (HPLC). Chromatograms were obtained with a reverse-phase high-performance liquid chromatography (RP-HPLC) system, an Agilent 1200 HPLC equipped with an autosampler, a binary pump, and a diode array detector (Agilent Technologies, Waldbronn, Germany).

The separation was performed on a Phenomenex[®] Gemini 5 μm C18 110A column under gradient

conditions. The mobile phase consisted of water and acetonitrile (CH_3CN), with the following gradient elution profile: method A (t_0 min 30% CH_3CN , t_8 min 80% CH_3CN , t_{22} min 90% CH_3CN , t_{25} min 30% CH_3CN) or method B (t_0 min 30% CH_3CN , t_8 min 80% CH_3CN , t_{14} min 30% CH_3CN).

The flow rate was maintained at 1 mL/min, and detection was performed at wavelengths of 210, 254, 280, and 310 nm to monitor the elution of compounds and assess purity.

Synthesis of Compounds 1a-3a

As reported in Scheme 3.8, 1-(4-(4-Acetyl-3-ethyl-5-methyl-1*H*-pyrrol-2-yl)thiazol-2-yl)-*N*-(2-(isoxazol-5-yl)ethyl) piperazine-2-carboxamide (5.88 mg, 0.013 mmol) was dissolved in 200 μL of dimethylformamide (DMF). 3-(2-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)ethoxy)propanoic acid (5.6 mg, 0.013 mmol), triethylamine (4 μL , 0.026 mmol), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (2.5 mg, 0.016 mmol) and hexafluorophosphate benzotriazole tetramethyl uronium (HBTU) (5.3 mg, 0.014 mmol) were added to the mixture at 0°C. The reaction was stirred at room temperature for 18 hours.

The solvent was evaporated and raw product was then purified by preparative thin-layer chromatography (PLC) eluting with dichloromethane/ethanol (9:1), obtaining final product **1a**.

The same procedure was applied for final products **2a** and **3a** by employing the following commercially available compounds: 3-(2-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)ethoxy)ethoxy)propanoic acid, 1-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)piperidine-4-carboxylic acid (Sigma Aldrich) (Scheme 3.8).

Data of Compound 1a

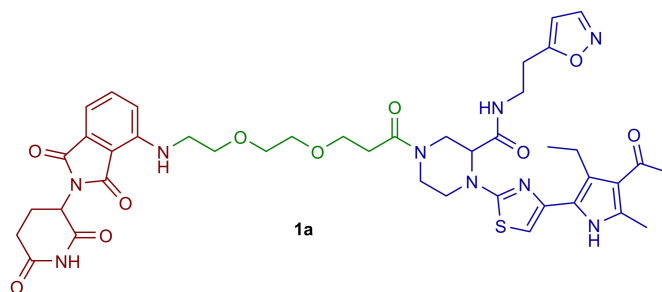


Figure 4.1: Product **1a**

Compound 1a: 1-(4-(4-acetyl-3-ethyl-5-methyl-1*H*-pyrrol-2-yl)thiazol-2-yl)-4-(3-(2-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)ethoxy)propanoyl)-*N*-(2-(isoxazol-5-yl)ethyl)piperazine-2-carboxamide

Yield: 30.6%

^1H NMR (400 MHz, CD_3OD) δ_{H} : 8.12 (s, 1H), 7.55-7.46 (m, 1H), 7.11-6.97 (m, 2H), 6.67-6.59 (m, 1H), 6.17-6.08 (m, 1H), 5.11-5.02 (m, 1H), 4.78-4.68 (m, 1H), 3.81-3.72 (m, 2H), 3.71-3.66 (m, 3H), 3.65-3.55 (m, 6H), 3.54-3.41 (m, 4H), 3.40-3.33 (m, 1H), 3.03-2.92 (m, 3H), 2.89-2.77 (m, 2H), 3.20-3.09 (m, 1H), 2.77-2.65 (m, 2H), 2.63-2.54 (m, 1H), 2.52 (s, 3H), 2.45 (s, 3H), 2.14-2.05 (m, 1H), 2.05-1.97 (m, 1H), 1.18-1.10 (m, 3H); (Figure S 8.1)

^{13}C NMR (100 MHz, CD_3OD) δ_{C} : 197.9, 174.7, 172.8, 171.8, 170.7, 166.4, 165.1, 151.6, 148.9, 148.2, 145.4, 142.5, 138.1, 137.4, 137.2, 133.8, 126.6, 124.5, 118.3, 112.1 (2C), 102.5, 101.2, 71.6, 70.6, 68.7, 68.4, 59.9, 50.2, 47.2, 46.4, 43.3, 38.6, 34.3, 32.2, 30.9, 28.8, 27.3, 20.1, 15.7, 15.2; (Figure S 8.2)

ESIMS (+): 872 $[\text{M}+\text{H}]^+$, 894 $[\text{M}+\text{Na}]^+$, ESIMS (-): m/z 870 $[\text{M}-\text{H}]^-$; calculated for $\text{C}_{42}\text{H}_{49}\text{N}_9\text{O}_{10}\text{S}$, 871.332;

Purity evaluation by analytical HPLC : 100.0%, t_{R} = 7.65 min (method A, λ = 254 nm)

Data of Compound 2a

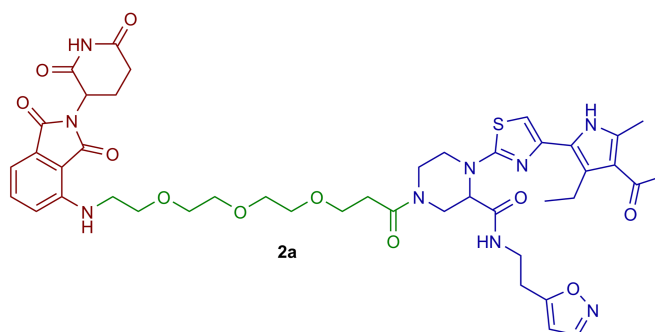


Figure 4.2: Product 2a

Compound 2a: 1-(4-(4-acetyl-3-ethyl-5-methyl-1H-pyrrol-2-yl)thiazol-2-yl)-4-(3-(2-(2-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)ethoxy)ethoxy)propanoyl)-N-(2-(isoxazol-5-yl)ethyl)piperazine-2-carboxamide

Yield: 36.3%

^1H NMR (400 MHz, CD_3OD) δ_{H} : 8.12 (s, 1H), 7.59-7.45 (m, 1H), 7.07-6.95 (m, 2H), 6.67-6.58 (m, 1H), 6.17-6.08 (m, 1H), 5.05 (dd, J = 12.0, 5.5 Hz, 1H), 4.79-4.70 (1H), 3.81-3.67 (m, 6H), 3.65-3.56 (m, 12H), 3.53-3.42 (m, 5H), 3.03-2.92 (m, 4H), 2.89-2.79 (m, 2H), 2.78-2.66 (m, 3H), 2.65-2.54 (m, 1H), 2.52 (s, 3H), 2.44 (s, 3H), 2.15-2.05 (m, 1H), 1.19-1.07 (m, 3H); (Figure S 8.3)

^{13}C NMR (100 MHz, CD_3OD) δ_{C} : 197.7, 174.7, 172.7, 171.7, 171.5, 170.9, 169.2, 151.5, 148.1, 137.3, 137.2, 133.8, 126.6, 124.4, 122.2, 120.6, 120.2, 118.3, 112.1, 111.2 (2C), 102.4, 102.3, 71.6 (2C), 71.5 (2C), 70.5, 68.5, 60.5, 60.0, 50.1, 47.1, 43.1, 41.9, 38.6, 32.2, 30.8, 28.7, 23.8, 20.1, 15.7, 15.1; (Figure S 8.4)

ESIMS (+): 916 $[\text{M}+\text{H}]^+$, ESIMS (-): m/z 914 $[\text{M}-\text{H}]^-$; calculated for $\text{C}_{44}\text{H}_{53}\text{N}_9\text{O}_{11}\text{S}$, 915.359; Purity evaluation by analytical HPLC : 100.0%, t_{R} = 7.67 min (method A, λ = 254 nm)

Data of Compound 3a

Compound 3a: 1-(4-(4-acetyl-3-ethyl-5-methyl-1H-pyrrol-2-yl)thiazol-2-yl)-4-(1-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)piperidine-4-carbonyl)-N-(2-(isoxazol-5-yl)ethyl)piperazine-2-carboxamide

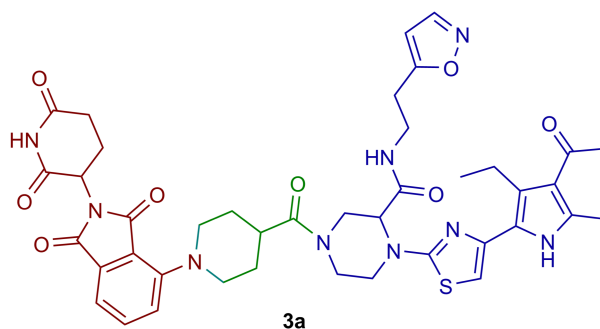


Figure 4.3: Product 3a

Yield: 15.3%

^1H NMR (400 MHz, CDCl_3) δ_{H} : 8.07 (s, 1H), 7.70-7.62 (m, 1H), 7.09-7.00 (m, 1H), 6.86-6.74 (m, 1H), 6.60-6.49 (m, 1H), 6.04-5.93 (m, 1H), 4.92 (dd, $J = 12.0, 5.5$ Hz, 1H), 4.68-4.60 (m, 1H), 4.37-4.28 (m, 1H), 4.05-3.89 (m, 2H), 3.67-3.29 (series of m, 4H), 2.93-2.88 (m, 3H), 2.85-2.66 (series of m, 3H), 2.54 (s, 3H), 2.45 (s, 3H), 2.15-2.08 (m, 1H), 2.00-1.89 (m, 2H), 1.88-1.77 (m, 2H), 1.22 (t, $J = 7.5$ Hz, 3H); (Figure S 8.5)

^{13}C NMR (100 MHz, CDCl_3) δ_{C} : 195.3, 174.0, 173.3, 171.1, 169.5, 168.4, 167.4, 155.3, 150.5 (2C), 134.6, 134.5, 129.2, 125.7, 125.5 (2C), 118.2, 118.0, 108.2 (2C), 101.7, 100.2, 60.5, 49.2, 47.4, 37.9, 37.6, 31.6, 31.1, 31.0, 28.1, 27.5, 26.7, 22.9, 21.2, 19.4, 17.4, 15.5, 14.9, 14.3; (Figure S 8.6)

ESIMS (+): 846 $[\text{M}+\text{Na}]^+$, ESIMS (-): m/z 822 $[\text{M}-\text{H}]^-$; calculated for $\text{C}_{41}\text{H}_{45}\text{N}_9\text{O}_8\text{S}$, 823.311.

Synthesis of Compound 1b

To a stirring solution of 1-(4-(4-acetyl-3-ethyl-5-methyl-1*H*-pyrrol-2-yl)thiazol-2-yl)-*N*-(2-(isoxazol-5-yl)ethyl)piperazine-2-carboxamide hydrochloride (10.4 mg, 0.02 mmol) in DMF, DIPEA (12 μL , 0.07 mmol) was added and the reaction was stirred at room temperature for 2 hours.

8-(((*S*)-1-((2*S*,4*R*)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-8-oxooctanoic acid (14.67 mg, 0.02 mmol), HBTU (9.47 mg, 0.025 mmol), and EDC (4.20 mg, 0.027 mmol) were then added sequentially to the mixture at 0°C. The solution was stirred at room temperature for 10 hours.

The raw product was poured into a NaHCO_3 saturated aqueous solution and extracted with ethyl acetate (3x). Combined organic phases were washed with NH_4Cl saturated aqueous solution (2x), brine (2x) and dried over Na_2SO_4 anhydrous.

The product was purified by silica column (dichloromethane/methanol 9:1) to furnish final product **1b** (Scheme 3.9).

Data of Compound 1b

Compound 1b: 1-(4-(4-acetyl-3-ethyl-5-methyl-1*H*-pyrrol-2-yl)thiazol-2-yl)-4-(8-(((*S*)-1-((2*S*,4*R*)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-8-oxooctanoyl)-*N*-(2-(isoxazol-5-yl)ethyl)piperazine-2-carboxamide

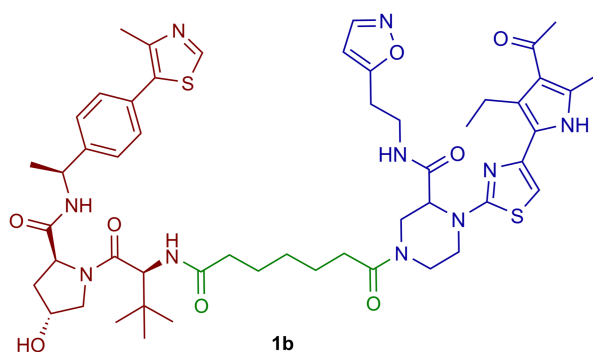


Figure 4.4: Product **1b**

Yield: 42.2%

^1H NMR (400 MHz, CDCl_3) δ_{H} : 9.33-9.20 (m, 1H), 8.67 (s, 1H), 8.00 (s, 1H), 7.56-7.45 (m, 1H), 7.42-7.34 (m, 4H), 6.76-6.66 (m, 1H), 6.55 (s, 1H), 6.41-6.25 (m, 1H), 5.96 (s, 1H), 5.14-5.03 (m, 1H), 4.98-4.86 (m, 1H), 4.73 (t, $J = 8.2$ Hz, 1H), 4.62-4.54 (m, 1H), 4.50 (s, 2H), 4.37-4.26 (m, 1H), 4.14-4.04 (m, 1H), 3.85-3.45 (series of m, 5H), 3.44-3.15 (m, 2H), 3.11-2.79 (m, 5H), 2.53 (s, 3H), 2.52-2.49 (m, 4H), 2.46 (s, 3H), 2.42-2.06 (m, 5H), 1.74-1.53 (m, 4H), 1.50-1.45 (m, 2H), 1.41-1.29 (m, 2H), 1.23 (t, $J = 7.1$ Hz, 3H), 1.23 (s, 9H); (Figure S 8.7)

^{13}C NMR (100 MHz, CDCl_3) δ_{C} : 195.2, 173.8, 172.6, 172.5, 171.2, 169.8, 169.4, 169.1, 150.3, 150.2, 148.9, 143.6, 143.3, 134.2, 131.6, 130.8, 129.5 (2C), 126.4 (2C), 125.4, 122.3, 122.1, 101.5, 99.9, 69.9, 59.4, 59.3, 58.6, 57.7, 56.9, 48.8, 46.6, 44.0, 40.2, 37.8, 35.9, 35.8, 35.0, 32.6, 32.4, 31.0 (2C), 28.4, 26.5 (3C), 22.2, 19.3, 16.1, 15.3, 14.7; (Figure S 8.8)

ESIMS (+): 1047 $[\text{M}+\text{Na}]^+$, ESIMS (-): m/z 1023 $[\text{M}-\text{H}]^-$; calculated for $\text{C}_{52}\text{H}_{68}\text{N}_{10}\text{O}_8\text{S}_2$, 1024,466;

Purity evaluation by analytical HPLC : 96.6%, $t_{\text{R}} = 7.55$ min (method A, $\lambda = 254$ nm)

Synthesis of Compounds 2b-7b

As reported in Scheme 3.10, (*S*,4*R*)-1-((*S*)-2-Amino-3,3-dimethylbutanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide hydrochloride (30mg, 0.064 mmol) was dissolved in DMF and DIPEA (35 μL , 0.20 mmol) was added to the mixture. The solution was stirred at room temperature for 1 hour.

6-(*tert*-Butoxy)-6-oxohexanoic acid (15.4 mg, 0.064 mmol), HBTU (26.6 mg, 0.07 mmol), and EDC (12 mg, 0.077 mmol) were then added sequentially to the mixture at 0°C. The reaction was stirred at room temperature for 10 hours.

The crude product was poured into a NaHCO_3 saturated aqueous solution and extracted with ethyl acetate (3x). Combined organic layers were washed with NH_4Cl saturated aqueous solution (2x), brine (2x) and dried over Na_2SO_4 anhydrous.

The product was purified by silica column (dichloromethane/methanol 9:1) to furnish precursors **10b**.

To a mixture of *tert*-butyl 6-(((*S*)-1-((2*S*,4*R*)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-6-oxohexanoate (**10b**) (28.8 mg, 0.047 mmol) in 1 mL of dichloromethane, trifluoroacetic acid (TFA) (100 μ L) was added drop-wise at 0°C. The reaction was stirred at room temperature for 24 hours.

The solvent was then evaporated and residue was purified by PLC (dichloromethane/methanol 9:1) to obtain compound **11b**.

To a stirring solution of 1-(4-(4-acetyl-3-ethyl-5-methyl-1*H*-pyrrol-2-yl)thiazol-2-yl)-*N*-(2-(isoxazol-5-yl)ethyl)piperazine-2-carboxamide hydrochloride (7.8 mg, 0.016 mmol) in DMF, DIPEA (9 μ L, 0.048 mmol) was added. The solution was stirred at room temperature for 2 hours.

6-(((*S*)-1-((2*S*,4*R*)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-6-oxohexanoic acid (**11b**) (8.7 mg, 0.016 mmol), HBTU (7.0 mg, 0.018 mmol), and EDC (3.1 mg, 0.02 mmol) were then added sequentially to the mixture at 0°C. The reaction was stirred at room temperature for 10 hours.

The crude product was poured into a NaHCO₃ saturated aqueous solution and extracted with ethyl acetate (3x). Combined organic layers were washed with NH₄Cl saturated aqueous solution (2x), brine (2x) and dried over Na₂SO₄ anhydrous.

The product was purified by PLC (dichloromethane/methanol 9:1) to furnish final compound **2b** (Scheme 3.10).

The same procedure was applied to obtain final products **3b**, **4b**, **5b**, **6b**, and **7b** by exploiting the following commercially available compounds: 10-(*tert*-butoxy)-10-oxododecanoic acid (**12b**, **13b** precursors), 12-(*tert*-butoxy)-12-oxododecanoic acid (**14b**, **15b** precursors), 3-(3-(*tert*-butoxy)-3-oxopropoxy)propanoic acid (**16b**, **17b** precursors), 3-(2-(3-(*tert*-butoxy)-3-oxopropoxy)ethoxy)propanoic acid (**18b**, **19b** precursors), 2,2-dimethyl-4-oxo-3,7,10,13-tetraoxahexadecan-16-oic acid (**20b**, **21b** precursors) (BLDPharm).

Data of Compound **2b**

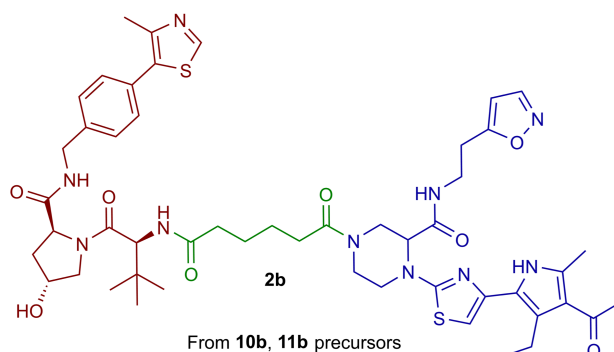


Figure 4.5: Product **2b**

Precursor 10b: *tert*-butyl 6-(((*S*)-1-((2*S*,4*R*)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-6-oxohexanoate

Yield: 73.3%

¹HNMR (400 MHz, CDCl₃) δ_{H} : 8.68 (s, 1H), 7.39-7.30 (m, 5H), 6.27 (d, J = 8.6 Hz, 1H), 4.72 (t, J = 7.8 Hz, 1H), 4.61 - 4.47 (m, 3H), 4.39-4.29 (m, 1H), 4.11-4.02 (m, 1H), 3.66-3.58

(m, 1H), 2.51 (s, 3H), 2.22-2.16 (m, 4H), 2.16-2.02 (m, 2H), 1.67-1.51 (m, 4H), 1.42 (s, 9H), 0.94 (s, 9H);

^{13}C NMR (100 MHz, CDCl_3) δ_{C} : 174.4, 173.1, 172.1, 170.8, 150.4, 148.8, 138.2, 131.7, 131.1, 129.6 (2C), 128.3 (2C), 80.5, 70.1, 58.6, 57.7, 56.8, 43.4, 36.2, 36.1, 35.2, 35.0, 28.2 (3C), 26.6 (3C), 25.1, 25.5, 16.2;

ESIMS (+): m/z 637 $[\text{M}+\text{Na}]^+$, ESIMS (-): m/z 613 $[\text{M}-\text{H}]^-$; calculated for $\text{C}_{32}\text{H}_{46}\text{N}_4\text{O}_6\text{S}$, 613.314.

Precursor 11b: 6-(((*S*)-1-((2*S*,4*R*)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-6-oxohexanoic acid

Yield: 51.2%

^1H NMR (400 MHz, CDCl_3) δ_{H} : 8.68 (s, 1H), 7.60 (t, $J = 5.8$, 1H), 7.37-7.38 (m, 4H), 7.09 (d, $J = 9.0$ Hz, 1H), 4.66 (t, $J = 7.7$ Hz, 1H), 4.62-4.48 (m, 3H), 4.35-4.27 (m, 1H), 4.10-4.02 (m, 1H), 3.70-3.64 (m, 1H), 2.49 (s, 3H), 2.36-2.12 (m, 6H), 1.66-1.50 (m, 4H), 0.96 (s, 9H);

^{13}C NMR (100 MHz, CDCl_3) δ_{C} : 176.7, 174.1, 171.9, 171.4, 150.6, 148.4, 138.5, 131.5, 130.8, 129.5 (2C), 128.0 (2C), 70.2, 59.3, 58.5, 57.3, 43.1, 37.1, 35.8, 35.5, 33.6, 26.5 (3C), 25.1, 24.3, 16.1;

ESIMS (+): m/z 581 $[\text{M}+\text{Na}]^+$, ESIMS (-): m/z 557 $[\text{M}-\text{H}]^-$; calculated for $\text{C}_{28}\text{H}_{38}\text{N}_4\text{O}_6\text{S}$, 558.251.

Compound 2b: 1-(4-(4-acetyl-3-ethyl-5-methyl-1*H*-pyrrol-2-yl)thiazol-2-yl)-4-(6-(((*S*)-1-((2*S*,4*R*)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-6-oxohexanoyl)-*N*-(2-(isoxazol-5-yl)ethyl)piperazine-2-carboxamide

Yield: 26.9%

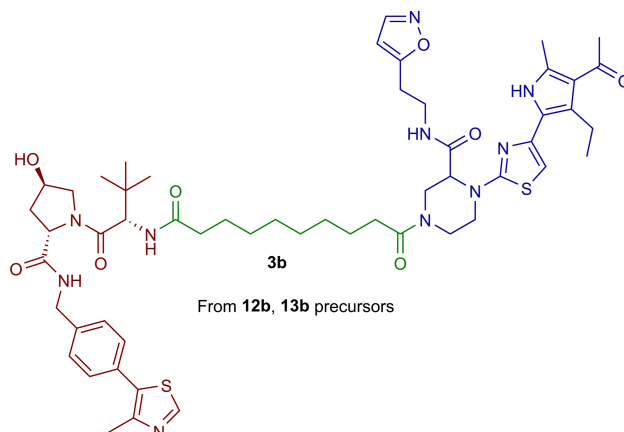
^1H NMR (400 MHz, CDCl_3) δ_{H} : 9.35-9.28 (m, 1H), 8.67 (s, 1H), 8.07 (s, 1H), 7.41-7.31 (m, 5H), 6.67-6.60 (m, 1H), 6.55 (s, 1H), 6.47-6.38 (m, 1H), 5.97 (s, 1H), 4.79-4.68 (m, 1H), 4.61-4.44 (m, 4H), 4.39-4.28 (m, 2H), 4.14-4.06 (m, 1H), 3.67-3.44 (m, 5H), 3.37-3.19 (m, 2H), 3.09-2.85 (m, 5H), 2.55 (s, 3H), 2.53-2.49 (m, 4H), 2.46 (s, 3H), 2.43-2.11 (m, 5H), 1.70-1.54 (m, 4H), 1.23-1.19 (m, 3H), 0.96 (s, 9H); (Figure S 8.9)

^{13}C NMR (100 MHz, CDCl_3) δ_{C} : 195.1, 173.5, 172.2, 172.0, 170.8, 169.4, 169.2, 169.1, 150.3, 150.2, 148.5, 143.6, 138.1, 134.2, 131.6, 131.0, 129.5 (2C), 128.1 (2C), 125.5, 122.3, 122.1, 101.4, 99.8, 70.1, 59.1, 58.5, 58.4, 56.8, 46.7, 43.9, 43.2, 40.2, 37.8, 36.1, 35.7, 34.9, 32.1, 31.0, 26.5, 26.4 (3C), 25.2, 23.8, 19.3, 16.1, 15.3, 14.7; (Figure S 8.10)

ESIMS (+): m/z 997 $[\text{M}+\text{H}]^+$, 1019 $[\text{M}+\text{Na}]^+$, ESIMS (-): m/z 995 $[\text{M}-\text{H}]^-$; calculated for $\text{C}_{50}\text{H}_{64}\text{N}_{10}\text{O}_8\text{S}_2$, 996,435;

Purity evaluation by analytical HPLC : 98.6%, $t_{\text{R}} = 7.61$ min (method A, $\lambda = 254$ nm).

Data of Compound 3b

**Figure 4.6:** Product 3b

Precursor 12b: *tert-butyl 10-(((S)-1-((2S,4R)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-10-oxodecanoate*

Yield: 60.1%

^1H NMR (400 MHz, CDCl_3) δ_{H} : 8.67 (s, 1H), 7.44-7.30 (m, 5H), 6.26-6.08 (m, 1H), 4.69 (t, $J = 7.9$ Hz, 1H), 4.59-4.47 (m, 1H), 4.39-4.29 (m, 1H), 4.11-4.01 (m, 1H), 3.67-3.55 (m, 1H), 2.50 (s, 3H), 2.49-2.42 (m, 1H), 2.21-2.08 (m, 5H), 1.66-1.49 (m, 4H), 1.42 (s, 9H), 1.32-1.23 (m, 8H), 0.93 (s, 9H);

^{13}C NMR (100 MHz, CDCl_3) δ_{C} : 173.7, 173.3, 171.8, 170.8, 150.3, 148.4, 138.1, 131.6, 130.9, 129.5 (2C), 128.0 (2C), 79.9, 70.0, 58.6, 57.4, 56.7, 43.2, 36.5, 35.9, 35.5, 35.0, 29.1, 29.0, 28.9 (2C), 28.1 (3C), 26.4 (3C), 25.6, 25.0, 16.0;

ESIMS (+): m/z 693 $[\text{M}+\text{Na}]^+$, ESIMS (-): m/z 669 $[\text{M}-\text{H}]^-$; calculated for $\text{C}_{36}\text{H}_{54}\text{N}_4\text{O}_6\text{S}$, 670.376.

Precursor 13b: *10-(((S)-1-((2S,4R)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-10-oxodecanoic acid*

Yield: 44.1%

^1H NMR (400 MHz, CDCl_3) δ_{H} : 8.70 (s, 1H), 7.39-7.31 (m, 5H), 6.94-6.87 (m, 1H), 4.67 (t, $J = 8.0$ Hz, 1H), 4.64-4.56 (m, 2H), 4.55-4.50 (m, 1H), 4.36-4.27 (m, 1H), 4.16-4.07 (m, 1H), 3.70-3.63 (m, 1H), 2.50 (s, 3H), 2.45-2.36 (m, 1H), 2.28 (t, $J = 7.1$ Hz, 2H), 2.23-2.13 (m, 3H), 1.68-1.50 (m, 4H), 1.36-1.23 (m, 8H), 0.95 (s, 9H);

^{13}C NMR (100 MHz, CDCl_3) δ_{C} : 177.0, 174.4, 172.1, 171.0, 150.3, 148.3, 138.2, 131.7, 130.7, 129.5 (2C), 127.9 (2C), 70.0, 59.0, 57.6, 57.0, 43.1, 36.4, 36.2, 35.2, 33.9, 28.8 (2C), 28.5, 28.4, 26.4 (3C), 25.4, 24.4, 15.9;

ESIMS (+): m/z 637 $[\text{M}+\text{Na}]^+$, ESIMS (-): m/z 613 $[\text{M}-\text{H}]^-$; calculated for $\text{C}_{32}\text{H}_{46}\text{N}_4\text{O}_6\text{S}$,

614.314.

Compound 3b: 1-(4-(4-acetyl-3-ethyl-5-methyl-1H-pyrrol-2-yl)thiazol-2-yl)-4-(10-(((S)-1-((2S,4R)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-10-oxodecanoyl)-N-(2-(isoxazol-5-yl)ethyl)piperazine-2-carboxamide

Yield: 36.7%

^1H NMR (400 MHz, CD_3OD) δ_{H} : 8.86 (s, 1H), 8.13 (s, 1H), 7.48-7.38 (m, 4H), 6.69-6.62 (m, 1H), 6.20-6.08 (m, 1H), 4.80-4.74 (m, 1H), 4.63 (s, 1H), 4.60-4.47 (m, 3H), 4.46-4.38 (m, 1H), 4.37-4.31 (m, 1H), 3.93-3.86 (m, 1H), 3.75-3.68 (m, 1H), 3.65-3.42 (m, 4H), 3.40-3.31 (m, 1H), 3.18-3.07 (m, 1H), 3.03-2.92 (m, 4H), 2.52 (s, 3H), 2.47 (s, 3H), 2.45-2.37 (m, 5H), 2.30-2.02 (m, 4H), 1.64-1.53 (m, 4H), 1.39-1.29 (m, 8H), 1.03 (s, 9H); (Figure S8.11)

^{13}C NMR (100 MHz, CD_3OD) δ_{C} : 197.8, 177.4, 176.1, 174.7, 174.5, 172.3, 172.1, 171.4, 152.8, 151.5, 148.9, 145.5, 144.3, 140.2, 137.4, 133.4, 131.5, 130.3 (2C), 128.9 (2C), 126.7, 102.4, 101.4, 71.1, 60.8, 60.6, 58.9, 58.8, 57.9, 47.1, 46.1, 45.6, 43.7, 41.9, 38.9, 38.5, 36.6, 33.7, 30.8, 30.4, 30.3, 30.1, 27.2, 27.1 (3C), 26.4, 26.2, 20.1, 15.9, 15.7, 15.1; (Figure S8.12)

ESIMS (+): m/z 1075 $[\text{M}+\text{Na}]^+$, ESIMS (-): m/z 1051 $[\text{M}-\text{H}]^-$; calculated for $\text{C}_{54}\text{H}_{72}\text{N}_{10}\text{O}_8\text{S}_2$, 1052.498.

Purity evaluation by analytical HPLC : 96.2%, $t_{\text{R}} = 8.67$ min (method B, $\lambda = 254$ nm).

Data of Compound 4b

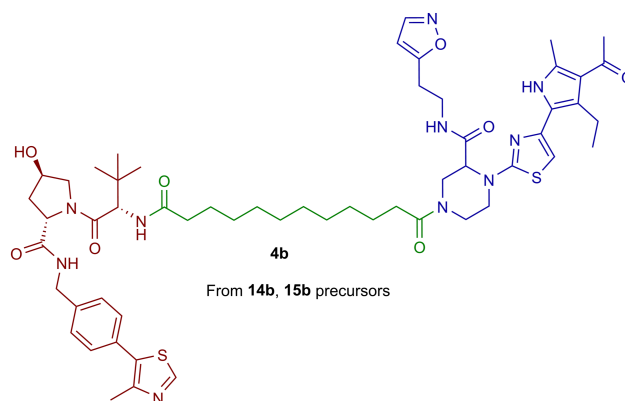


Figure 4.7: Product 4b

Precursor 14b: *tert*-butyl 12-(((S)-1-((2S,4R)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-12-oxododecanoate

Yield 69.2%

^1H NMR (400 MHz, CDCl_3) δ_{H} : 8.66 (s, 1H), 7.43-7.29 (m, 5H), 6.34-6.24 (m, 1H), 4.65 (t, $J = 7.8$ Hz, 1H), 4.58-4.45 (m, 3H), 4.36-4.28 (m, 1H), 4.05-3.98 (m, 1H), 3.64-3.57 (m, 1H), 2.49 (s, 3H), 2.46-2.36 (m, 1H), 2.20-2.13 (m, 5H), 1.61-1.48 (m, 4H), 1.42 (s, 9H), 1.28-1.21 (m,

12H), 0.92 (s, 9H);

^{13}C NMR (100 MHz, CDCl_3) δ_{C} : 173.9, 173.4, 171.7, 171.0, 150.4, 148.4, 138.2, 131.6, 130.8, 129.5 (2C), 128.0 (2C), 79.9, 69.8, 58.7, 57.2, 56.8, 43.2, 38.6, 36.5, 35.6, 35.2, 29.4, 29.3, 29.2 (2C), 29.1, 29.0, 28.1 (3C), 26.4 (3C), 25.8, 25.0, 15.9;

ESIMS (-): m/z 697 $[\text{M}-\text{H}]^-$; calculated for $\text{C}_{38}\text{H}_{58}\text{N}_4\text{O}_6\text{S}$, 698.408.

Precursor 15b: 12-(((*S*)-1-((2*S*,4*R*)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-12-oxododecanoic acid

Yield: 44.1%

^1H NMR (400 MHz, CDCl_3) δ_{H} : 8.70 (s, 1H), 7.39-7.32 (m, 4H), 7.27-7.22 (m, 1H), 7.06-6.99 (m, 1H), 4.68 (t, $J = 8.1$ Hz, 1H), 3.72-3.62 (m, 1H), 2.51 (s, 3H), 2.46-2.39 (m, 1H), 2.31 (t, $J = 6.8$ Hz, 2H), 2.25-2.12 (m, 3H), 1.63-1.52 (m, 4H), 1.35-1.22 (m, 12H), 0.95 (s, 9H);

^{13}C NMR (100 MHz, CDCl_3) δ_{C} : 177.0, 174.5, 172.2, 170.9, 150.4, 148.3, 138.1, 131.6, 130.8, 129.4 (2C), 127.9 (2C), 70.0, 59.0, 57.5, 57.1, 43.2, 36.4, 36.1, 35.2, 33.8, 28.8, 28.4, 28.2 (2C), 28.1, 28.0, 26.4, (3C), 25.1, 24.4, 15.9;

ESIMS (+): m/z 665 $[\text{M}+\text{Na}]^+$, ESIMS (-): m/z 641 $[\text{M}-\text{H}]^-$; calculated for $\text{C}_{34}\text{H}_{50}\text{N}_4\text{O}_6\text{S}$, 642.345.

Compound 4b: 1-(4-(4-acetyl-3-ethyl-5-methyl-1*H*-pyrrol-2-yl)thiazol-2-yl)-4-(12-(((*S*)-1-((2*S*,4*R*)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-12-oxododecanoyl)-*N*-(2-(isoxazol-5-yl)ethyl)piperazine-2-carboxamide

Yield: 22.4%

^1H NMR (400 MHz, CD_3OD) δ_{H} : 8.86 (s, 1H), 8.13 (s, 1H), 7.49-7.37 (m, 4H), 6.71-6.60 (m, 1H), 6.19-6.06 (m, 1H), 4.80-4.74 (m, 1H), 4.66-4.61 (m, 1H), 4.60-4.48 (m, 3H), 4.46-4.40 (m, 1H), 4.38-4.31 (m, 1H), 4.30-4.21 (m, 1H), 3.92-3.86 (m, 1H), 3.83-3.77 (m, 1H), 3.75-3.68 (m, 1H), 3.65-3.42 (m, 4H), 3.20-3.07 (m, 1H), 3.04-2.91 (m, 4H), 2.52 (s, 3H), 2.47 (s, 3H), 2.45-2.34 (m, 5H), 2.33-2.16 (m, 3H), 2.12-2.02 (m, 1H), 1.66-1.52 (m, 4H), 1.39-1.25 (m, 12H), 1.17-1.09 (m, 3H), 1.02 (s, 9H); (Figure S 8.13)

^{13}C NMR (100 MHz, CD_3OD) δ_{C} : 199.3, 177.6, 176.2, 175.9, 173.9, 172.9, 154.3, 153.1, 150.5, 146.9, 141.8, 138.9, 134.8, 133.0, 131.8 (2C), 130.5 (2C), 128.3, 125.9, 123.7, 103.9, 102.9, 102.7, 72.6, 62.3, 62.0, 61.2, 60.5, 59.4, 48.6, 47.5, 47.1, 45.1, 43.4, 40.4, 40.2, 38.2, 38.1, 35.4, 35.2, 32.4, 32.0, 31.9, 31.8, 28.8, 28.6 (3C), 27.9, 27.7, 21.6, 17.3, 16.6; (Figure S 8.14)

ESIMS (+): m/z 1103 $[\text{M}+\text{Na}]^+$, ESIMS (-): m/z 1079 $[\text{M}-\text{H}]^-$; calculated for $\text{C}_{56}\text{H}_{76}\text{N}_{10}\text{O}_8\text{S}_2$, 1080.529.

Purity evaluation by analytical HPLC : 98.3%, $t_{\text{R}} = 9.22$ min (method B, $\lambda = 254$ nm).

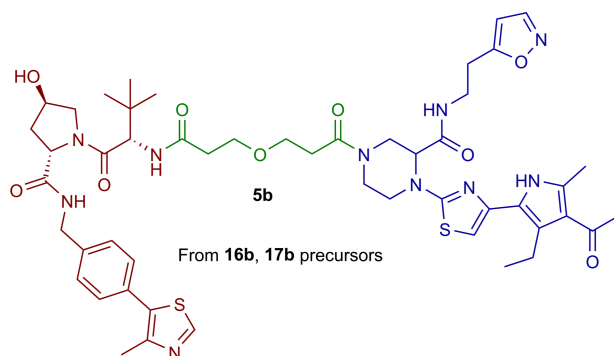


Figure 4.8: Product 5b

Data of Compound 5b

Precursor 16b: *tert-butyl 3-(3-(((S)-1-((2S,4R)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-3-oxopropoxy)propanoate*

Yield: 60.9%

^1H NMR (400 MHz, CDCl_3) δ_{H} : 8.72 (s, 1H), 7.51-7.42 (m, 1H), 7.40-7.29 (m, 4H), 6.99-6.90 (m, 1H), 4.72 (t, $J = 7.8$ Hz, 1H), 4.61-4.42 (m, 3H), 4.39-4.29 (m, 1H), 4.12-4.02 (m, 1H), 3.76-3.57 (m, 5H), 2.52 (s, 3H), 2.51-2.47 (m, 3H), 2.48 (t, $J = 5.4$ Hz, 2H), 2.17-2.07 (m, 1H), 1.43 (s, 9H), 0.94 (s, 9H);

^{13}C NMR (100 MHz, CDCl_3) δ_{C} : 171.9, 171.8, 170.9, 170.5, 150.4, 148.1, 138.3, 131.8, 130.7, 129.5 (2C), 128.2 (2C), 80.8, 70.1, 66.8, 66.7, 58.8, 57.7, 56.6, 43.2, 36.7, 36.1, 35.9, 28.1 (3C), 26.3 (3C), 15.9;

ESIMS (+): m/z 653 $[\text{M}+\text{Na}]^+$, ESIMS (-): m/z 629 $[\text{M}-\text{H}]^-$; calculated for $\text{C}_{32}\text{H}_{46}\text{N}_4\text{O}_7\text{S}$, 630.309.

Precursor 17b: *3-(3-(((S)-1-((2S,4R)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-3-oxopropoxy)propanoic acid*

Yield: 43.1%

^1H NMR (400 MHz, CDCl_3) δ_{H} : 8.83 (s, 1H), 7.56-7.47 (m, 1H), 7.42-7.31 (m, 4H), 7.12-7.02 (m, 1H), 4.77-4.70 (m, 1H), 4.65-4.49 (m, 3H), 4.37-4.28 (m, 1H), 4.14-4.03 (m, 1H), 3.78-3.59 (m, 5H), 2.56-2.48 (m, 5H), 2.48-2.43 (m, 2H), 2.41-2.34 (m, 1H), 2.27-2.19 (m, 1H), 0.95 (m, 1H);

^{13}C NMR (100 MHz, CDCl_3) δ_{C} : 174.1, 172.0, 171.7, 171.2, 151.1, 138.4, 132.4, 130.1, 129.6, 129.4 (2C), 128.2 (2C), 70.4, 66.6, 66.3, 58.9, 57.5, 57.0, 43.1, 36.5 (2C), 35.6, 34.9, 26.3 (3C), 15.4;

ESIMS (+): m/z 597 $[\text{M}+\text{Na}]^+$, ESIMS (-): m/z 573 $[\text{M}-\text{H}]^-$; calculated for $\text{C}_{28}\text{H}_{38}\text{N}_4\text{O}_7\text{S}$, 574.246.

Compound 5b: Data of 1-(4-(4-acetyl-3-ethyl-5-methyl-1H-pyrrol-2-yl)thiazol-2-yl)-4-(3-(3-(((S)-1-((2S,4R)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-3-oxopropoxy)propanoyl)-N-(2-(isoxazol-5-yl)ethyl)piperazine-2-carboxamide

Yield: 26.9%

^1H NMR (400 MHz, CD_3OD) δ_{H} : 8.83 (s, 1H), 8.17-8.10 (m, 1H), 7.47-7.31 (m, 4H), 6.68-6.61 (m, 1H), 6.20-6.08 (m, 1H), 4.80-4.70 (m, 1H), 4.69-4.64 (m, 1H), 4.62-4.52 (m, 1H), 4.51-4.37 (m, 3H), 4.36-4.27 (m, 1H), 4.26-4.03 (m, 1H), 3.93-3.83 (m, 1H), 3.82-3.65 (m, 6H), 3.64-3.41 (m, 4H), 3.03-2.90 (m, 4H), 2.89-2.55 (series of m, 2H), 2.55-2.51 (m, 1H), 2.50-2.48 (m, 5H), 2.47-2.40 (m, 6H), 2.28-2.13 (m, 1H), 2.13-2.04 (m, 1H), 1.16-1.09 (m, 3H), 1.03 (s, 9H); (Figure S 8.15)

^{13}C NMR (100 MHz, CD_3OD) δ_{C} : 197.8, 174.5, 173.9, 172.6, 172.2, 171.4, 152.8, 151.6, 149.1, 145.5, 140.1, 137.4, 133.5, 131.6, 130.4, 130.4 (2C), 128.9 (2C), 126.9, 124.4, 122.4, 102.4, 101.5, 101.3, 71.1, 68.2, 68.1, 60.7, 58.9, 58.1, 47.0, 43.7, 42.0, 39.0, 38.7, 37.3, 36.8, 34.6, 34.5, 30.9, 27.3, 27.1 (2C), 20.1, 15.8, 15.2; (Figure S 8.16)

ESIMS (+): m/z 1035 $[\text{M}+\text{Na}]^+$, ESIMS (-): m/z 1011 $[\text{M}-\text{H}]^-$; calculated for $\text{C}_{50}\text{H}_{64}\text{N}_{10}\text{O}_9\text{S}_2$, 1012.430

Purity evaluation by analytical HPLC : 98.5%, t_{R} = 7.53 min (method A, λ = 254 nm).

Data of Compound 6b

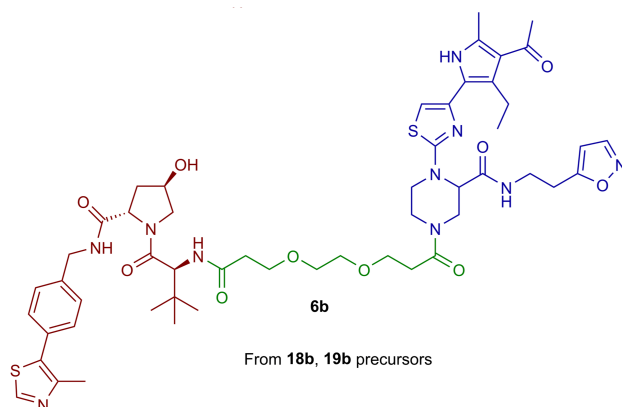


Figure 4.9: Product 6b

Precursor 18b: *tert*-butyl 3-(2-(3-(((S)-1-((2S,4R)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-3-oxopropoxy)ethoxy)propanoate

Yield: 55.8%

^1H NMR (400 MHz, CDCl_3) δ_{H} : 8.68 (s, 1H), 7.48 (t, J = 5.7 Hz, 1H), 7.39-7.32 (m, 4H), 7.04 (d, J = 8.6 Hz, 1H), 4.74 (t, J = 7.9 Hz, 1H), 4.60-4.48 (m, 2H), 4.47-4.42 (m, 1H), 4.39-4.29 (m, 1H), 4.14-4.06 (m, 1H), 3.74-3.66 (m, 4H), 3.64-3.57 (m, 5H), 2.52 (s, 3H), 2.51-2.45

(m, 4H), 2.17-2.02 (m, 2H), 1.43 (s, 9H), 0.94 (s, 9H);

^{13}C NMR (100 MHz, CDCl_3) δ_{C} : 172.2, 171.8, 170.9, 170.8, 150.3, 148.5, 138.2, 131.6, 130.9, 129.5 (2C), 128.1 (2C), 80.7, 70.4, 70.2, 70.1, 67.1, 66.8, 58.4, 57.8, 56.6, 43.2, 36.7, 36.2, 35.9, 34.7, 28.1 (3C), 26.4 (3C), 16.0;

ESIMS (+): m/z 697 $[\text{M}+\text{Na}]^+$, ESIMS (-): m/z 673 $[\text{M}-\text{H}]^-$; calculated for $\text{C}_{34}\text{H}_{50}\text{N}_4\text{O}_8\text{S}$, 674.335.

Precursor 19b: 10-(((*S*)-1-((2*S*,4*R*)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-10-oxodecanoic acid

Yield: 39.4%

^1H NMR (400 MHz, CDCl_3) δ_{H} : 8.70 (s, 1H), 7.39-7.31 (m, 5H), 6.94-6.87 (m, 1H), 4.67 (t, $J = 8.0$ Hz, 1H), 4.64-4.56 (m, 2H), 4.55-4.50 (m, 1H), 4.36-4.27 (m, 1H), 4.16-4.07 (m, 1H), 3.70-3.63 (m, 1H), 2.50 (s, 3H), 2.45-2.36 (m, 1H), 2.28 (t, $J = 7.1$ Hz, 2H), 2.23-2.13 (m, 3H), 1.68-1.50 (m, 4H), 1.36-1.23 (m, 8H), 0.95 (s, 9H);

^{13}C NMR (100 MHz, CDCl_3) δ_{C} : 175.6, 172.8, 171.9, 171.2, 150.3, 148.4, 138.4, 131.7, 130.8, 129.4 (2C), 128.1 (2C), 70.3, 70.2, 69.9, 67.1, 66.6, 59.0, 58.3, 57.0, 43.1, 36.7, 36.3, 34.9 (2C), 26.4 (3C), 16.1;

ESIMS (+): m/z 641 $[\text{M}+\text{Na}]^+$, ESIMS (-): m/z 617 $[\text{M}-\text{H}]^-$; calculated for $\text{C}_{32}\text{H}_{46}\text{N}_4\text{O}_6\text{S}$, 618.272.

Compound 6b: 1-(4-(4-acetyl-3-ethyl-5-methyl-1*H*-pyrrol-2-yl)thiazol-2-yl)-4-(10-(((*S*)-1-((2*S*,4*R*)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-10-oxodecanoyl)-*N*-(2-(isoxazol-5-yl)ethyl)piperazine-2-carboxamide

Yield: 34.5%

^1H NMR (400 MHz, CD_3OD) δ_{H} : 8.85 (s, 1H), 8.13 (s, 1H), 7.48-7.34 (m, 4H), 6.71-6.60 (m, 1H), 6.19-6.08 (m, 1H), 4.80-4.72 (m, 1H), 4.69-4.63 (m, 1H), 4.61-4.52 (m, 1H), 4.51-4.46 (m, 2H), 4.38-4.31 (m, 1H), 3.91-3.85 (m, 1H), 3.84-3.65 (m, 7H), 3.64-3.55 (m, 6H), 3.54-3.43 (m, 2H), 3.18-3.05 (m, 1H), 3.04-2.93 (m, 4H), 2.89-2.54 (m, 3H), 2.54-2.48 (m, 4H), 2.46 (s, 3H), 2.44 (s, 3H), 2.26-2.17 (m, 1H), 2.11-2.03 (m, 1H), 1.18-1.09 (m, 3H), 1.03 (s, 9H); (Figure S 8.17)

^{13}C NMR (100 MHz, CD_3OD) δ_{C} : 197.8, 174.5, 173.8, 172.7, 172.1, 171.4, 152.8, 151.6, 149.1, 145.5, 145.4, 140.3, 137.4, 133.7, 131.6, 130.3 (2C), 129.0 (2C), 126.7, 124.4, 122.3, 102.5, 101.5, 101.3, 71.5, 71.1, 68.5, 68.2, 60.8, 60.5, 60.0, 58.8, 58.0, 47.2, 46.4, 43.7, 41.9, 38.9, 37.4, 36.8, 34.3, 30.8, 27.3, 27.0, (3C), 20.1, 15.8, 15.7, 15.1; (Figure S 8.18)

ESIMS (+): m/z 1079 $[\text{M}+\text{Na}]^+$, ESIMS (-): m/z 1055 $[\text{M}-\text{H}]^-$; calculated for $\text{C}_{52}\text{H}_{68}\text{N}_{10}\text{O}_8\text{S}_2$, 1056.456.

Purity evaluation by analytical HPLC : 98.8%, $t_{\text{R}} = 7.44$ min (method B, $\lambda = 254$ nm).

Data of Compound 7b

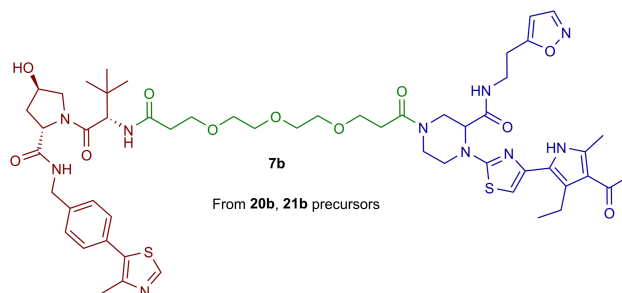


Figure 4.10: Product 7b

Precursor 20b: *tert-butyl (S)-15-((2S,4R)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidine-1-carbonyl)-16,16-dimethyl-13-oxo-4,7,10-trioxa-14-azaheptadecanoate*

Yield: 60.9%

^1H NMR (400 MHz, CDCl_3) δ_{H} : 8.73 (s, 1H), 7.53-7.44 (m, 1H), 7.41-7.30 (m, 4H), 7.09-7.07 (m, 1H), 4.72 (t, $J = 7.8$ Hz, 1H), 4.60-4.42 (m, 3H), 4.39-4.29 (m, 1H), 4.13-4.04 (m, 1H), 3.73-3.66 (m, 4H), 3.65-3.54 (m, 10H), 2.52 (s, 3H), 2.51-2.44 (m, 5H), 1.43 (s, 9H), 0.94 (s, 9H);

^{13}C NMR (100 MHz, CDCl_3) δ_{C} : 172.1, 171.8, 170.9 (2C), 150.5, 149.0, 138.4, 131.9, 130.6, 129.5, (2C), 128.1 (2C), 80.5, 70.5, 70.4 (2C), 70.3, 70.1, 67.1, 66.9, 58.4, 57.7, 56.6, 43.1, 36.7, 36.2, 35.9, 34.8, 28.0 (3C), 26.4 (3C), 15.8;

ESIMS (+): m/z 741 $[\text{M}+\text{Na}]^+$, ESIMS (-): m/z 717 $[\text{M}-\text{H}]^-$; calculated for $\text{C}_{36}\text{H}_{54}\text{N}_4\text{O}_9\text{S}$, 718.361.

Precursor 21b: *(S)-15-((2S,4R)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidine-1-carbonyl)-16,16-dimethyl-13-oxo-4,7,10-trioxa-14-azaheptadecanoic acid*

Yield: 25.8%

^1H NMR (400 MHz, CDCl_3) δ_{H} : 8.76 (s, 1H), 7.43-7.31 (m, 6H), 4.71 (t, $J = 7.8$ Hz, 1H), 4.67-4.58 (m, 2H), 4.36-4.29 (m, 2H), 4.20-4.12 (m, 1H), 3.81-3.69 (m, 4H), 4.68-3.54 (m, 4H), 3.68-3.54 (m, 10H), 2.61-2.49 (m, 6H), 2.47-2.39 (m, 1H), 2.27-2.17 (m, 1H), 0.97 (s, 9H);

^{13}C NMR (100 MHz, CDCl_3) δ_{C} : 178.9, 174.4, 171.9, 171.1, 150.7, 147.9, 138.6, 131.9, 130.5, 129.5 (2C), 128.1 (2C), 70.5, 70.4 (2C), 70.3, 70.2, 67.1, 66.7, 58.9, 58.4, 57.9, 57.1, 43.2, 36.7, 36.4, 35.2, 26.4 (3C), 15.7;

ESIMS (+): m/z 685 $[\text{M}+\text{Na}]^+$, ESIMS (-): m/z 661 $[\text{M}-\text{H}]^-$; calculated for $\text{C}_{32}\text{H}_{46}\text{N}_4\text{O}_9\text{S}$, 662.299.

Compound 7b: *1-(4-(4-acetyl-3-ethyl-5-methyl-1H-pyrrol-2-yl)thiazol-2-yl)-4-((S)-15-((2S,4R)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidine-1-carbonyl)-16,16-dimethyl-13-oxo-4,7,10-trioxa-14-azaheptadecanoyl)-N-(2-(isoxazol-5-yl)ethyl)piperazine-2-carboxamide*

Yield: 40.6%

^1H NMR (400 MHz, CD_3OD) δ_{H} : 8.85 (s, 1H), 8.14 (s, 1H), 7.49-7.36 (m, 4H), 6.71-6.62 (m, 1H), 6.19-6.10 (m, 1H), 4.98-4.92 (m, 1H), 4.81-4.72 (m, 1H), 4.65-4.63 (m, 1H), 4.60-4.52 (m, 2H), 4.52-4.45 (m, 2H), 4.38-4.30 (m, 1H), 3.92-3.84 (m, 1H), 3.83-3.67 (m, 6H), 3.65-3.54 (m, 10H), 3.53-3.45 (m, 2H), 3.16-3.05 (m, 1H), 3.04-2.92 (m, 4H), 2.86-2.75 (m, 1H), 2.71-2.56 (m, 2H), 2.57-2.50 (m, 4H), 2.46 (s, 3H), 2.43 (s, 3H), 2.52-2.16 (m, 1H), 2.12-2.05 (m, 1H), 1.18-1.09 (m, 3H), 1.03 (s, 9H); (Figure S 8.19)

^{13}C NMR (100 MHz, CD_3OD) δ_{C} : 197.8, 174.5, 173.4, 172.7, 172.1, 171.5, 152.8, 151.6, 149.1, 145.4, 140.2, 137.5, 133.4, 131.5, 130.5, 130.4 (2C), 128.9 (2C), 126.7, 124.3, 122.2, 102.5, 101.5, 101.3, 71.5, 71.4 (2C), 71.3, 68.3, 68.2, 60.8, 60.5, 58.8, 58.0, 47.1, 43.7, 38.9, 37.4, 36.7, 34.6, 34.3, 30.8, 30.6, 27.3, 27.0 (3C), 20.1, 15.9, 15.1; (Figure S 8.20)

ESIMS (+): m/z 1123 $[\text{M}+\text{Na}]^+$, ESIMS (-): m/z 1099 $[\text{M}-\text{H}]^-$; calculated for $\text{C}_{54}\text{H}_{72}\text{N}_{10}\text{O}_{11}\text{S}_2$, 1100.482

Purity evaluation by analytical HPLC : 98.1%, $t_{\text{R}} = 7.38$ min (method A, $\lambda = 254$ nm).

Synthesis of Compound 8b

As described in Scheme 3.11, (2*S*,4*R*)-1-((*S*)-2-Amino-3,3-dimethylbutanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide hydrochloride (60 mg, 0.13 mmol) was dissolved in DMF and DIPEA (66.8 μL , 0.39 mmol) was added. The solution was stirred at room temperature for 2 hours.

To the mixture were then added 3-(4-(*tert*-butoxycarbonyl)piperazin-1-yl)benzoic acid (40 mg, 0.13 mmol), HBTU (53 mg, 0.14 mmol) and EDC (24 mg, 0.15 mmol) at 0°C, stirring at room temperature for 10 hours.

The crude product was poured into a NaHCO_3 saturated aqueous solution and extracted with ethyl acetate (3x). The combined organic layers were washed with NH_4Cl saturated aqueous solution (2x), brine (2x) and dried over Na_2SO_4 anhydrous.

The precursor was purified by silica column (dichloromethane/methanol 9:1) to give **22b**.

tert-butyl 4-(3-(((*S*)-1-((2*S*,4*R*)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)carbamoyl)phenyl)piperazine-1-carboxylate (**22b**) (74.3 mg) was dissolved in 334 μL of tetrahydrofuran (THF), then 666 μL of 3M HCl aqueous solution were carefully added at 0°C to reach 2 M final HCl concentration. The reaction was stirred at room temperature for 18 hours.

The solution was concentrated *in vacuo* to give a residue that was poured in 10% K_2CO_3 aqueous solution, extracted with dichloromethane (3x), and dried over Na_2SO_4 anhydrous. The raw product was purified by PLC (dichloromethane/methanol 9:1) to obtain compound **23b**.

To a mixture of 4-(3-(((*S*)-1-((2*S*,4*R*)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)carbamoyl)phenyl)piperazine-1-carboxylic acid (**23b**) (39.18 mg, 0.063 mmol) in DMF, DIPEA (33 μL , 0.19 mmol), 4-(*tert*-butoxy)-4-oxobutanoic acid (11 mg, 0.063 mmol), HBTU (26.3 mg, 0.070 mmol), EDC (11.7 mg, 0.076 mmol) were added sequentially at 0°C. The reaction was stirred at room temperature for 12 hours.

The crude product was poured into a NaHCO_3 saturated aqueous solution and extracted with ethyl acetate (3x). The combined organic layers were washed with NH_4Cl saturated aqueous solution (2x), brine (2x) and dried over Na_2SO_4 anhydrous.

The precursor was purified by silica column (dichloromethane/methanol 9:1) to give **24**.

tert-Butyl 4-(4-(3-(((*S*)-1-((2*S*,4*R*)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)carbamoyl)phenyl)piperazin-1-yl)-4-oxobutanoate (**24b**) (17.2 mg, 0.02 mmol) was dissolved in 1 mL of dichloromethane, then trifluoroacetic acid (100 μ L) was added drop-wise at 0°C. The reaction was stirred at room temperature for 24 hours. The solvent was then evaporated and the residue was purified by PLC (dichloromethane/methanol 9:1) to obtain precursor **25b**.

To a stirring solution of 1-(4-(4-acetyl-3-ethyl-5-methyl-1*H*-pyrrol-2-yl)thiazol-2-yl)-N-(2-(isoxazol-5-yl)ethyl)piperazine-2-carboxamide hydrochloride (4.2 mg, 0.009 mmol) in DMF, DIPEA (5 μ L, 0.027 mmol) was added. The solution was stirred at room temperature for 2 hours.

4-(4-(3-(((*S*)-1-((2*S*,4*R*)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)carbamoyl)phenyl)piperazin-1-yl)-4-oxobutanoic acid (**25b**) (6.6 mg, 0.009 mmol), HBTU (3.79 mg, 0.01 mmol), and EDC (1.7 mg, 0.01 mmol) were then added sequentially to the mixture at 0°C. The reaction was stirred at room temperature for 10 hours.

The raw product was poured into a NaHCO₃ saturated aqueous solution and extracted with ethyl acetate (3x). Combined organic layers were washed with NH₄Cl saturated aqueous solution (2x), brine (2x) and dried over Na₂SO₄ anhydrous. The product was purified by PLC (dichloromethane/methanol 9:1) to furnish final compound **8b** (Scheme 3.11).

Data of Compound **8b**

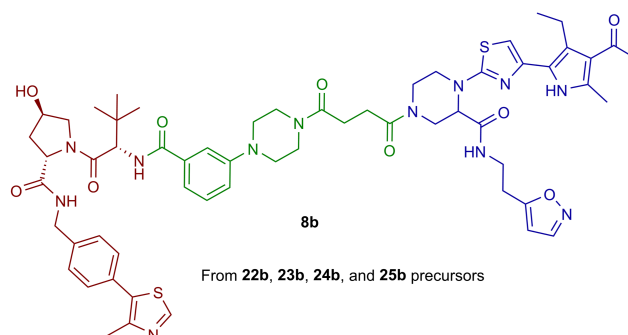


Figure 4.11: Product **8b**

Precursor **22b:** *tert*-butyl 4-(3-(((*S*)-1-((2*S*,4*R*)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)carbamoyl)phenyl)piperazine-1-carboxylate

Yield: 80.8%

¹HNMR (400 MHz, CDCl₃) δ _H: 8.62 (s, 1H), 7.42-7.29 (m, 6H), 7.26-7.22 (m, 1H), 7.14-7.09 (m, 1H), 7.06-7.01 (m, 1H), 6.83-6.77 (m, 1H), 4.75-4.65 (m, 2H), 4.56-4.47 (m, 2H), 4.37-4.27 (m, 1H), 4.13-4.06 (m, 1H), 3.68-3.63 (m, 1H), 3.59-3.52 (m, 4H), 3.20-3.11 (m, 4H), 2.49 (s, 3H), 2.46-2.38 (m, 1H), 2.12-2.03 (m, 1H), 1.46 (s, 9H), 0.99 (s, 9H);

^{13}C NMR (100 MHz, CDCl_3) δ_{C} : 171.6, 170.8 (2C), 167.9, 154.6, 151.3, 150.3, 148.3, 138.2, 134.7, 131.7, 130.8, 129.5 (2C), 128.1 (2C), 119.4, 117.9 (2C), 115.6, 80.0, 70.1, 58.6, 57.8, 56.8, 49.1 (2C), 43.2 (2C), 36.1, 35.6, 28.4 (3C), 26.5 (3C), 16.0;

ESIMS (+): m/z 719 $[\text{M}+\text{H}]^+$, 741 $[\text{M}+\text{Na}]^+$, ESIMS (-): m/z 717 $[\text{M}-\text{H}]^-$; calculated for $\text{C}_{38}\text{H}_{50}\text{N}_6\text{O}_6\text{S}$, 718.905.

Precursor 23b: *Data of 4-(3-(((S)-1-((2S,4R)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)carbamoyl)phenyl)piperazine-1-carboxylic acid*

Yield: 61.3%

^1H NMR (400 MHz, CDCl_3) δ_{H} : 8.71 (s, 1H), 7.59-7.50 (m, 1H), 7.42-7.28 (m, 6H), 7.17-7.12 (m, 1H), 7.04-6.97 (m, 1H), 6.93-6.85 (m, 1H), 4.82-4.72 (m, 2H), 4.61-4.52 (m, 2H), 4.44-4.34 (m, 1H), 4.20-4.13 (m, 1H), 3.78-3.68 (m, 1H), 3.27-3.11 (m, 4H), 3.09-2.99 (m, 4H), 2.55 (s, 3H), 2.51-2.42 (m, 1H), 2.19-2.12 (m, 1H), 1.05 (s, 9H);

^{13}C NMR (100 MHz, CDCl_3) δ_{C} : 171.6, 170.6 (2C), 168.9, 151.7, 150.3, 148.1, 138.2, 134.5, 131.6, 130.9, 129.4 (2C), 128.1 (2C), 119.4, 117.2 (2C), 114.9, 70.0, 58.7, 57.9, 56.9, 49.3 (2C), 45.6, 43.2, 36.3, 35.5, 26.4 (3C), 15.4;

ESIMS (+): 685 $[\text{M}+\text{Na}]^+$, ESIMS (-): m/z 661 $[\text{M}-\text{H}]^-$; calculated for $\text{C}_{34}\text{H}_{42}\text{N}_6\text{O}_6\text{S}$, 662.799.

Precursor 24b: *tert-butyl 4-(4-(3-(((S)-1-((2S,4R)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)carbamoyl)phenyl)piperazin-1-yl)-4-oxobutanoate*

Yield: 35.2%

^1H NMR (400 MHz, CDCl_3) δ_{H} : 8.69 (s, 2H), 7.43-7.28 (m, 7H), 7.20-7.15 (m, 1H), 7.07-7.02 (m, 1H), 6.77-6.71 (m, 1H), 4.80-4.69 (m, 2H), 4.64-4.55 (m, 2H), 4.41-4.31 (m, 1H), 4.22-4.16 (m, 1H), 3.81-3.74 (m, 2H), 3.70-3.63 (m, 3H), 3.27-3.16 (m, 4H), 2.67-2.57 (m, 5H), 2.52 (s, 3H), 2.11-2.09 (m, 1H), 1.45 (s, 9H), 1.00 (s, 9H);

^{13}C NMR (100 MHz, CDCl_3) δ_{C} : 172.4, 171.7, 170.8, 170.0, 167.9, 151.2, 150.3, 148.5, 138.1, 131.6, 130.9, 128.5 (2C), 129.4, 128.1 (2C), 119.5, 117.8 (2C), 115.5, 80.5, 70.1, 58.7, 57.9, 56.8, 49.1, 48.8, 45.1, 43.3, 41.5, 38.4, 36.1, 35.6, 30.3, 28.1 (3C), 26.1 (3C), 16.2;

ESIMS (+): m/z 775 $[\text{M}+\text{H}]^+$, 797 $[\text{M}+\text{Na}]^+$, ESIMS (-): m/z 773 $[\text{M}-\text{H}]^-$; calculated for $\text{C}_{34}\text{H}_{42}\text{N}_6\text{O}_6\text{S}$, 774.968.

Precursor 25b: *4-(4-(3-(((S)-1-((2S,4R)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)carbamoyl)phenyl)piperazin-1-yl)-4-oxobutanoic acid*

Yield: 41.8%

^1H NMR (400 MHz, CDCl_3) δ_{H} : 8.69 (s, 1H), 7.51-7.44 (m, 1H), 7.39-7.28 (m, 5H), 7.24-7.20 (m, 1H), 7.18-7.13 (m, 1H), 7.05-6.96 (m, 1H), 4.82-4.74 (m, 1H), 4.68 (t, $J = 7.8$ Hz, 1H), 4.58-4.47 (m, 2H), 4.39-4.29 (m, 1H), 4.16-4.07 (m, 1H), 3.71-3.64 (m, 3H), 3.62-3.54 (m, 2H), 3.21-3.03 (m, 4H), 2.69-2.56 (m, 4H), 2.49 (s, 3H), 2.41-2.32 (m, 1H), 2.21-2.07 (m, 1H), 1.02 (s, 9H);

^{13}C NMR (100 MHz, CDCl_3) δ_{C} : 177.9, 174.5, 174.1, 173.1, 171.1, 154.0, 153.4, 151.3, 141.1, 137.5, 134.5, 133.7, 132.4 (2C), 130.9 (2C), 122.9, 120.9 (2C), 118.2, 73.0, 61.8, 60.9, 59.9, 51.9, 51.6, 48.1, 46.0, 44.5, 39.3, 38.5, 32.3, 30.8, 29.3 (3C), 18.8;

ESIMS (+): 741 $[\text{M}+\text{Na}]^+$, ESIMS (-): m/z 717 $[\text{M}-\text{H}]^-$; calculated for $\text{C}_{37}\text{H}_{46}\text{N}_6\text{O}_7\text{S}$, 718.862.

Compound 8b: 1-(4-(4-acetyl-3-ethyl-5-methyl-1H-pyrrol-2-yl)thiazol-2-yl)-4-(4-(4-(3-(((S)-1-((2S,4R)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)carbamoyl)phenyl)piperazin-1-yl)-4-oxobutanoyl)-N-(2-(isoxazol-5-yl)ethyl)piperazine-2-carboxamide

Yield: 31.8%

^1H NMR (400 MHz, CD_3OD) δ_{H} : 8.87 (s, 1H), 8.13 (s, 1H), 7.49-7.38 (m, 5H), 7.21-7.13 (m, 1H), 6.71-6.63 (m, 1H), 6.18-6.09 (m, 1H), 4.78-4.71 (m, 1H), 4.65-4.47 (m, 4H), 4.42-4.21 (m, 2H), 4.01-3.94 (m, 1H), 3.93-3.80 (m, 2H), 3.79-3.70 (m, 5H), 3.67-3.57 (m, 2H), 3.55-3.45 (m, 2H), 3.44-3.36 (m, 1H), 3.26-3.18 (m, 2H), 3.17-3.09 (m, 1H), 3.06-2.93 (m, 4H), 2.85-2.62 (m, 5H), 2.55-2.51 (m, 3H), 2.47 (s, 3H), 2.44 (s, 3H), 2.28-2.19 (m, 1H), 2.16-2.07 (m, 1H), 1.19-1.13 (m, 3H), 1.11 (s, 9H); (Figure S 8.21)

^{13}C NMR (100 MHz, CD_3OD) δ_{C} : 197.9, 174.4, 173.2, 172.8, 172.4, 171.5, 170.2, 152.8, 152.7, 151.6, 149.1, 145.5, 140.3, 137.4, 136.2, 133.5, 131.5, 130.5 (2C), 129.4, 128.9 (2C), 126.7, 124.4, 122.3, 120.9, 119.7 (2C), 116.3, 102.4, 101.4, 101.2, 71.0, 60.9, 59.3, 58.2, 47.1, 46.9, 46.0, 45.4, 43.7, 43.5, 42.9, 42.0, 39.0, 38.7, 38.6, 37.3, 30.8, 29.0, 28.8, 27.3, 27.1 (3C), 20.1, 15.8, 15.1; (Figure S 8.22)

ESIMS (+): 1179 $[\text{M}+\text{Na}]^+$, ESIMS (-): m/z 1155 $[\text{M}-\text{H}]^-$ calculated for $\text{C}_{59}\text{H}_{72}\text{N}_{12}\text{O}_9\text{S}_2$, 1156.499.

Purity evaluation by analytical HPLC : 100.0%, t_{R} = 8.09 min (method A, λ = 254 nm).

4.3 CRBN and DDB1 Δ B Proteins Expression and Purification

CRBN and DDB1 Δ B MiniPrep

50 μL of XL1 Blue competent cells were transformed with 0.5 μL of N-terminal His₆ CRBN (pAJ075_hsCRBN, Addgene plasmid #124214) and HisSpy DDB1 Δ B (pESF140_HisSpy_DD B1 Δ B, Addgene plasmid #124213) DNA. Samples were incubated in ice for 30 minutes and heatshocked for 40 seconds at 42°C and 2 minutes in ice. 250 μL of LB were added to the cells and samples were incubated at 37°C, 650 rpm shaking for 1 hour in Eppendorf ThermoMixer C. 100 μL of the mixtures were then inoculated in 10 mL of LB media supplemented with Ampicillin (100 $\mu\text{g}/\text{mL}$) and incubated overnight at 37°C, shaking in LOM-80 MRC shaker.

The mixtures were centrifuged in Allegratm 25R Beckman Coulter at 3000x g, 4°C for 30 minutes. Plasmids were then extracted using E.Z.N.A.[®] Plasmid DNA Mini Kit, Omega Bio-tek.

Sf9 Transfection

Sf9 insect cells were seeded at the final concentration of 1.00×10^6 cells/well on a 6-well plate and incubated at 27°C for 2 hours in IPP 55 Memmert incubator. Transfection mixes were

prepared by combining 2 μg of CRBN or DDB1 Δ B DNA, 0.5 μg of dsRED linearized DNA, 10 μL of Transporter 5[®] Transfection Reagent (Polysciences), and 100 μL of 1x PBS. Solutions were incubated at room temperature for 20 minutes. 1 mL of media was then carefully removed from the 6-well plate and transfection mixes were added drop-wise to the wells. Cells were incubated for 4.5 hours at 27°C, subsequently, 1 mL of fresh media was added carefully to the wells. Plate was incubated at 27°C for two weeks, following the cell growth and production of dsRED marker protein.

Insect Cells Infection

Transfected cells were collected and centrifuged at 1000 xg for 10 minutes in Allegra[™] X-22R Beckman Coulter. Supernatant was collected and 2% v/v of FBS filtered at 0.22 μm was added. P0 was stored at 4°C.

Viral vectors were further amplified by adding 1 mL of P0 to 40 mL of Sf9 cells at the density of 0.8×10^9 cells/mL. The cell culture was incubated at 27°C, 130 rpm shaking in CERTOMAT BS-1 (Sartorius) for 4 days monitoring the cell count and size. When the cells stopped proliferating and their diameter reached 16.9 - 17.1 μm the cells were centrifuged at 1000 xg for 10 minutes in Allegra[™] X-22R Beckman Coulter. Supernatant was collected and 2% v/v of filtered FBS (0.22 μm) was added (P1). P1 was stored at 4°C. The proteins were co-expressed in H5 cells (Expression Systems) using 1 L bottles (Vitlab). 4 x 1 L of cell culture at density of 1.0×10^6 cells/mL supplemented with 50 μM zinc acetate were infected with 5 mL of P1 CRBN and 5 mL P1 DDB1 Δ B and incubated at 27°C, 150 rpm shaking for 3 days in Innova S44i incubators (Eppendorf).

Cells were then centrifuged for 1 hour, 1000 xg, at 4°C on Avanti[®], J-26 XP, Beckman Coulter centrifuge. The supernatant was discarded, and pellets were resuspended on 50 mM Tris pH 8, 500 mM NaCl, 10% glycerol, 10 mM imidazole, 2 mM DTT buffer containing protease inhibitor mix (Roche) and Benzonase nuclease. Cells were lysed using ultrasonic processor by applying 35% amplitude, 15 second impulse, 45 pause for 10 minutes. Lysate was then centrifuged for 1 hour, 30 000 xg, at 4°C on Avanti[®], J-26 XP, Beckman Coulter centrifuge to eliminate cell debris.

CRBN and DDB1 Δ B Purification

The cell lysate was loaded on Ni-NTA agarose resin (Qiagen) (2 mL) previously equilibrated with 50mM Tris pH 8, 500 mM NaCl, 10% glycerol, 10 mM imidazole, 2 mM DTT buffer and transferred to a gravity-flow column. Complex was eluted using 50 mM Tris pH 8, 500 mM NaCl, 10% glycerol, 300 mM imidazole, 2 mM DTT. Collected fractions were then loaded on 5 mL HiTrap HP Q (Cytiva) anion exchange column and purified on AKTA pure FPLC purifier system. Column was equilibrated using 25 mM Hepes pH 7.4, 1mM TCEP buffer and elution was performed through a linear gradient to 1 M NaCl of the same buffer. In the last step, CRBN-DDB1 Δ B complex was injected on Superdex200 16/60 Prep grade column and eluted with 50 mM HEPES pH 7.4, 200 mM NaCl, 1 mM TCEP buffer. Collected fractions were concentrated to 22.5 mg/mL, flash frozen and stored at -80°C.

4.4 VHL and EloB/C Proteins Expression and Purification

VHL and EloB/C MiniPrep

VHL (pIVM_02, Addgene plasmid #204500) and EloB/C (pIVM_26, Addgene plasmid #204501) were transformed on DH5alpha competent cells and DNA was extracted using EasyPure[®]

MiniPrep Kit, Transgen Biotech.

50 μL of BL21(DE3) competent cells were transformed with 1.5 μL of VHL and EloB/C plasmids. The sample was incubated in ice for 30 minutes and heatshocked for 45 seconds at 42°C and left on ice for more than 2 minutes. 450 μL of LB was added to the cells, and samples were incubated at 37°C, 190 rpm shaking for 1 hour. 100 μL of the sample were streaked on LB Agar plates supplemented with Ampicillin (100 $\mu\text{g}/\text{mL}$) and Streptomycin (50 $\mu\text{g}/\text{mL}$). Plates were left overnight at 37°C to allow colony formation.

VHL and EloB/C Expression

A single colony was picked and inoculated on 200 mL LB media supplemented with Ampicillin (100 $\mu\text{g}/\text{mL}$) and Streptomycin (50 $\mu\text{g}/\text{mL}$). Preculture was grown overnight at 37°C, 190 rpm shaking on New Brunswick Innova[®] 44 incubator.

20 mL of preculture (1/50) were taken and transferred to 1 L x 2 of LB media supplemented with Ampicillin (100 $\mu\text{g}/\text{mL}$) and Streptomycin (50 $\mu\text{g}/\text{mL}$). The culture was grown at 37°C, 190 rpm shaking in New Brunswick Innova[®] 44 incubator until OD₆₀₀ reached 0.7. VHL-EloB/C expression was induced with 0.3 mM IPTG, and culture was grown at 24°C, 190 rpm shaking overnight.

Cells were collected and centrifuged at 5000 rpm, using a Beckman Avanti J-26S XP centrifuge, at 4°C. Pellets were resuspended on 20 mM Tris pH 8.0, 500 mM NaCl, 10 mM imidazole, supplemented with complete, EDTA-free, protease inhibitor mix (Roche), and lysed by ultrasonic processor by applying 35% amplitude, 15 second impulse, 45 pause for 10 minutes. Cell lysate was incubated for 30 minutes at room temperature with 10 $\mu\text{g}/\text{mL}$ DNaseI and 10 mM MgCl₂. Cell debris was removed by centrifugation for 40 minutes at 20 000 rpm, using a Beckman Avanti J-26S XP centrifuge, at 4°C.

VHL and EloB/C Purification

VHL-EloB/C complex was purified using the AKTA pure FPLC purifier system.

Supernatant was filtered (0.22 μm) and loaded on 5 mL HisTrap HP column. System was equilibrated in 20 mM Tris pH 8.0, 500 mM NaCl, 10 mM imidazole and proteins were eluted using a 45% Tris pH 8.0, 500 mM NaCl, 500 mM imidazole buffer. The collected fractions were dialyzed using 10MWCO snakeskin dialysis membrane (ThermoFisher) in presence of TEV for His₆ tag cleavage. Proteins were left overnight at 4°C. The complex was loaded again on 5 mL HisTrap HP column and eluted in the flow through. VHL-EloB/C were diluted ten times with 20 mM Bis-Tris pH 7.0, 1 mM DTT buffer and loaded on 1 mL HiTrap, CaptoQ anion exchange column. Elution was performed by using a linear gradient to 1M NaCl of the same buffer. In the last purification step, the complex was injected on a Superdex75 16/60 Prep grade column, equilibrated in 20 mM Bis-Tris pH 7.0, 150 mM NaCl, 1 mM DTT. Collected fractions were concentrated to 19 mg/mL, snap frozen and stored at -80°C.

4.5 BAZ2A/2B Expression and Purification

BAZ2A bromodomain expression was induced by adding 0.2 mM IPTG to *E. coli* culture and grown overnight at 18°C. Bromodomain was then loaded on Ni-NTA column and eluted with an increasing concentration of imidazole. 6xHis-tagged protein was removed using TEV protease and protein was further purified with a second IMAC. In the last step, protein was purified using Superdex 75-16/600 column, concentrated at 23 mg/mL and frozen in liquid nitrogen.

BAZ2B bromodomain expression was induced using 0.5 mM IPTG and grown overnight at 18°C.

Protein was purified using IMAC and eluted with a linear gradient of imidazole. The tag was removed with TEV protease, and bromodomains were purified with a second IMAC and a SEC using Superdex 75-16/600 column. Purified protein was concentrated at 15 mg/mL and frozen in liquid nitrogen.

4.6 Structural Characterization

Crystallization

The CRBN-DDB1 Δ B complex (50 μ M) was combined with BAZ2A or BAZ2B bromodomains (50 μ M) and PROTACs **1a**, **2a**, and **3a** (200 μ M). The mixtures were incubated at 4°C for one hour prior to crystallization trials. Crystallization experiments were performed using vapour diffusion in 2-subwell plates (Hampton Research) with either the Mosquito (sptlabtech) or Oryx4 (Douglas Instruments) setup. Protein-to-precipitant ratios of 2:1 and 1:1 were tested. A variety of crystallization screens were employed, including Morpheus (Molecular Dimensions), Proplex (Molecular Dimensions), JCSG (Jena Bioscience), and SG1 (Molecular Dimensions). Plates were incubated at 20°C and crystals were forming within a few weeks in D2, D11, and H12 wells from SG1 screening. Positive conditions were presenting the following compositions:

- 0.1 M Sodium citrate pH 5.5, 20% w/v PEG 3000
- 0.1 M Sodium acetate pH 4.6, 8% w/v PEG 4000
- 0.1 M Sodium HEPES pH 7.5, 4.3 M Sodium chloride

Promising conditions identified from the SG1 screen were further optimized by fine-tuning the pH, PEG, and NaCl concentrations to enhance crystal formation.

The crystals were cryo-protected with a solution of identical composition as the crystallization buffer except for increased PEG and ethylene glycol (42% to 54%) concentration, then frozen in liquid nitrogen.

VHL-EloB/C complex (160 μ M) was mixed with BAZ2A or BAZ2B bromodomains (160 μ M) and VHL-based PROTACs (**b** series, 240 μ M, ethylene glycol). The mixture was dissolved in a buffer containing 20 mM HEPES (pH 7.5), 150 mM NaCl, and 1 mM TCEP, and incubated at 4°C for 1 hour. Crystallization trials were conducted via vapour diffusion in 2-subwell plates (Hampton Research) using the Oryx4 (Douglas Instruments) setup. Plates were incubated at both 20°C and 4°C, with crystal formation observed within one week. Promising conditions (B3, B5, B11, and E2 from the SG1 screen) featured the following compositions: 0.2 M $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$, 0.1 M Bis-Tris, pH 5.5, 25% PEG 3350; 0.2 M $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$, 20% PEG 3350; 0.2 M NH_4Cl , 20% PEG 3350; 25% PEG 3350.

- 0.1 M Bis-Tris, 0.2 pH 5.5, 0.2 M Magnesium chloride hexahydrate, 25% w/v PEG 3350
- 0.2 M Calcium chloride dihydrate, 20% w/v PEG 3350
- 0.2 M Ammonium chloride, 20% w/v PEG 3350
- 25% w/v PEG3350

Conditions identified as promising were further optimized by adjusting pH, PEG, and salt concentrations to improve crystal formation.

The crystals were then cryo-protected with a solution of identical composition as the crystallization buffer except for increased PEG and ethylene glycol (40% to 50%, including PROTACs solvent) concentration, then frozen in liquid nitrogen.

Data Collection and Structure Solution

Data were collected at the ESRF synchrotron (Grenoble, France), beamline ID30-A3 and Elettra synchrotron (Trieste, Italy), XRD2 beamline. XDS and AIMLESS were used for data integration, reduction, and scaling, while molecular replacement was performed with PHASER using as search models PDB 9BEE (trimmed of beta-propeller B) for DDB1 and PDB 5T35 (devoid of BRD4 and the PROTAC molecule) for VHL/EloB/EloC. Structure refinement was performed through alternating cycles of manual rebuilding in COOT and automated refinement with PHENIX. The refinement included the use of X, Y, and Z coordinates, as well as individual B-factors. Thermal motion was modeled isotropically. For the VHL/EloB/EloC complex, non-crystallographic symmetry (NCS) restraints were applied during refinement.

Chapter 5

Conclusions and Future Perspectives

In this work, we finely analyze the role of BAZ2A in cancer as a putative therapeutic target. Starting from the classical inhibition approach, we investigated the effect of impairing BAZ2A function by blocking Kac recognition through a competitive inhibitor targeting BAZ2A bromodomain. By testing previously developed inhibitors in prostate cancer cell lines, we proved the inefficiency of simply inhibiting BAZ2A bromodomain, demonstrating that interfering with the Kac recognition did not affect the functioning of the entire BAZ2A complex. At the same time, we reconfirmed the potential of eliminating the overall complex in promoting an antiproliferative effect, resulting in the only strategy successfully impairing BAZ2A-mediated tumorigenic features (Figure 5.1, 1.). Therefore, we envisioned another strategy to circumvent BAZ2A pathological function, involving the exploitation of PROTAC technology, capable of inducing POI elimination through the cellular machinery devoted to degradation. We designed degraders based on high-resolution structures encompassing our hit compound bound to BAZ2A bromodomain and released ternary complex structures (Figure 5.1, 2.). In terms of chemical structure, all degraders hold the same POI ligand, and they differentiate for the E3 ligase recruiter moiety and linker region connecting the two main components. In detail, we decided to promote target elimination by fostering CRBN and VHL ligases-mediated degradation while we explored different lengths, polarity and rigidity in linker structure (Figure 5.1, 3.). Once we synthesized a proper number of degraders, we decided to proceed with the structural characterization and the degradation activity assessment. The former consisted of solving ternary complex structures by X-ray crystallographic analysis. Unfortunately, although proteins were successfully produced and formed crystals under supersaturation conditions, we did not obtain ternary complex structures. Notably, CRBN-based ternary complex resulted in the crystallization of only DDB1 due to an increased abundance of such protein compared to the other components. In the case of VHL, crystals were composed of binary complexes involving the interaction between the E3 ligase and developed PROTACs. Although we did not achieve ternary complex structure solutions, failed crystallization attempts may provide insights for future trials (Figure 5.1, 4.). At the same time, developed PROTACs were tested for their ability to induce BAZ2A degradation by western blotting. Preliminary experiments showed that only VHL-based PROTACs mediated BAZ2A degradation, indicating degraders with longer alkylic linkers as the most active and holding promises for late-stage prostate cancer treatment (Figure 5.1, 5.).

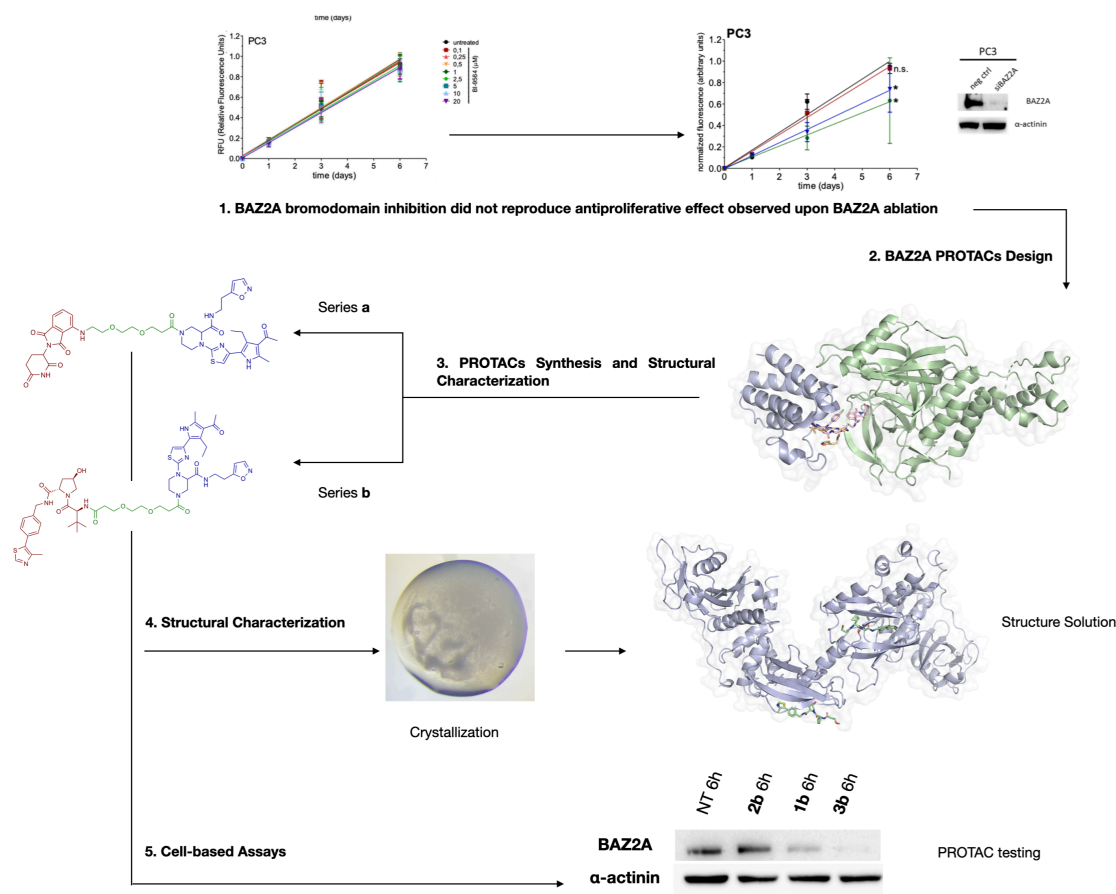


Figure 5.1: Scheme summarizes the different steps of the study, starting from experiments showing the inefficiency of BAZ2A bromodomain inhibition while proving the potential of BAZ2A ablation (1.). Therefore, the study proceeded with the design of PROTAC molecules capable of inducing BAZ2A degradation (2.). We then move to synthesize designed degraders, subdividing them according to their capability to recruit CRBN or VHL E3 ligases (3.). We also attempted structural characterization of ternary complexes through X-ray crystallographic analysis (4.), while testing developed degraders for their ability to degrade the target protein (5.).

In the future, further experiments will be necessary to confirm the successful elimination of BAZ2A and establish an accurate degradation profile. High-throughput screening methods, like HiBit (Promega), will facilitate rapid screening of all synthesized degraders. Simultaneously, we will conduct additional crystallization attempts based on previous trials. The results of the structural characterization, combined with cell-based screening, will provide insights for future structural modifications to enhance PROTAC degradation.

Lastly, developed degraders will be tested for their ability to produce an antiproliferative effect on prostate cancer cell lines, as previously observed upon siRNA-mediated BAZ2A elimination. Additionally, synthesized PROTACs will be evaluated for their ability to promote the ablation also of BAZ2B, which has been proven beneficial in slowing the ageing process.

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Chapter 6

Author Contributions

The contributions of each author to this thesis are as follows:

- Eleonora Parolin: Conceived the research idea, designed and performed experiments, analyzed the data, wrote the manuscript.
- Graziano Lolli: Conceived the research idea, assisted in experimental design, provided critical guidance throughout the project, reviewed and revised the manuscript.
- Ines Mancini: Supervised and provided critical guidance in the chemical synthesis, reviewed and revised the manuscript.
- Paola Storici: Supervised and provided critical guidance in insect cell protein expression.
- Giulia Cazzanelli: Performed and provided assistance in cell-based assays and western blot analysis.
- Marta Stefania Semrau: Supervised and provided assistance in insect cell protein expression, performed mass photometer analysis.

Chapter 7

Acknowledgments

I would like to express my deepest gratitude to my supervisor, Professor Graziano Lolli, for his invaluable guidance, unwavering support, and for generously sharing his expertise as a crystallographer. His insightful advice and encouragement have been crucial throughout these years.

I am also sincerely grateful to all the members of the Laboratory of Bioorganic Chemistry, especially Professor Ines Mancini, whose exceptional supervision and inspiring dedication to her work have had a profound impact on my research. My heartfelt thanks go to Mario Rossi and Sandro Gadotti for their technical support.

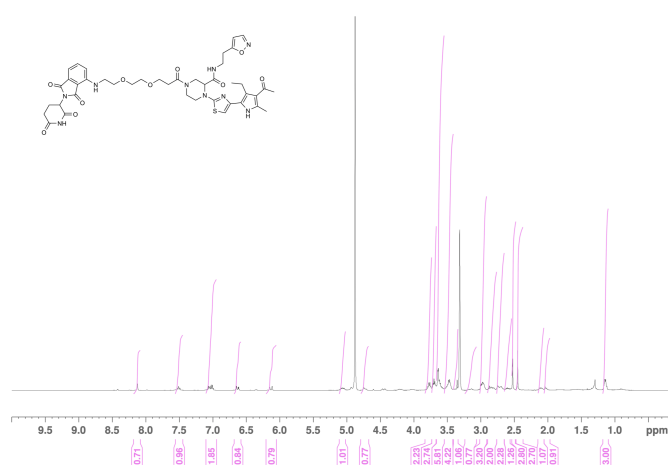
I would like to extend my thanks to the members of the Protein Facility at Elettra Synchrotron, Trieste, for their warm welcome and hospitality. In particular, I am grateful to Dr. Paola Storici and Dr. Marta Stefania Semrau for their invaluable assistance and support with protein production.

A special thank you goes to Dr. Lavinia Carlini and Dr. Giulia Cazzanelli for patiently sharing their knowledge and expertise, which have been instrumental in my progress.

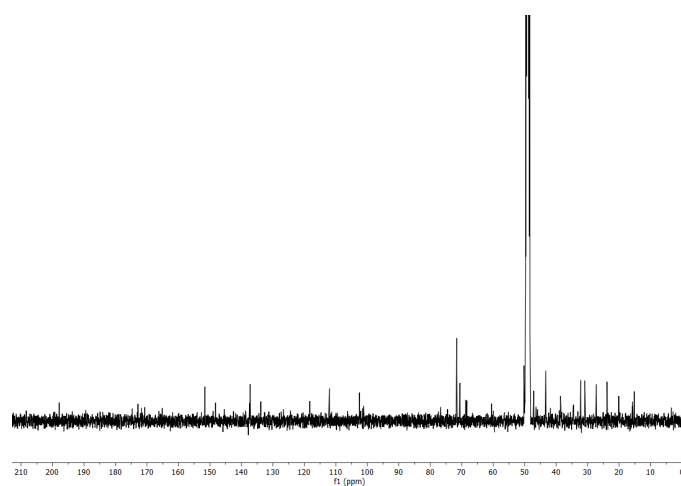
Lastly, I would like to express my profound gratitude to Nicole Innocenti, Roberto Miani, Jacopo Vigna and Francesco Trenti for accompanying me on this challenging journey. Your friendship, encouragement, and support have made this experience truly unforgettable.

Chapter 8

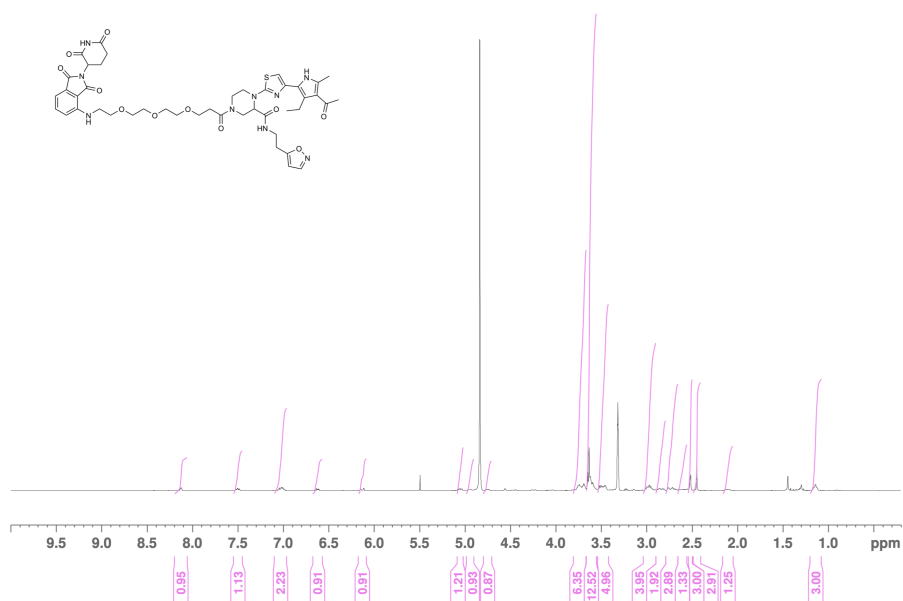
Supplementary



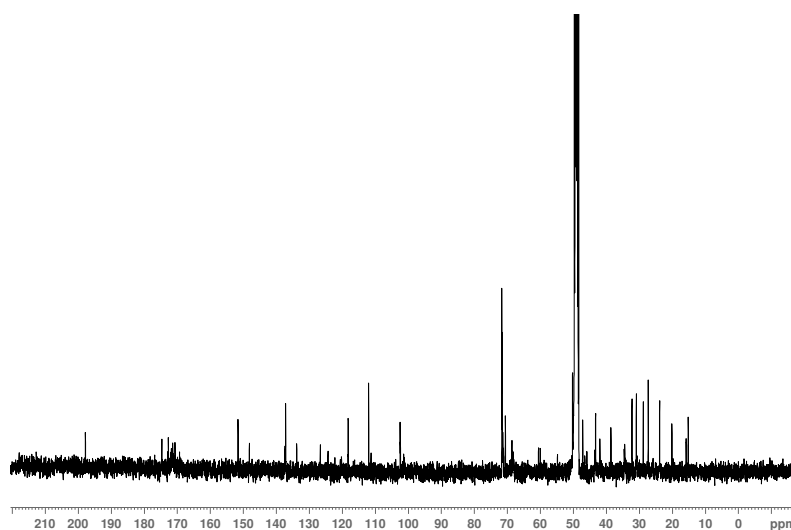
S 8.1: ¹H NMR Spectrum of 1a in CD₃OD



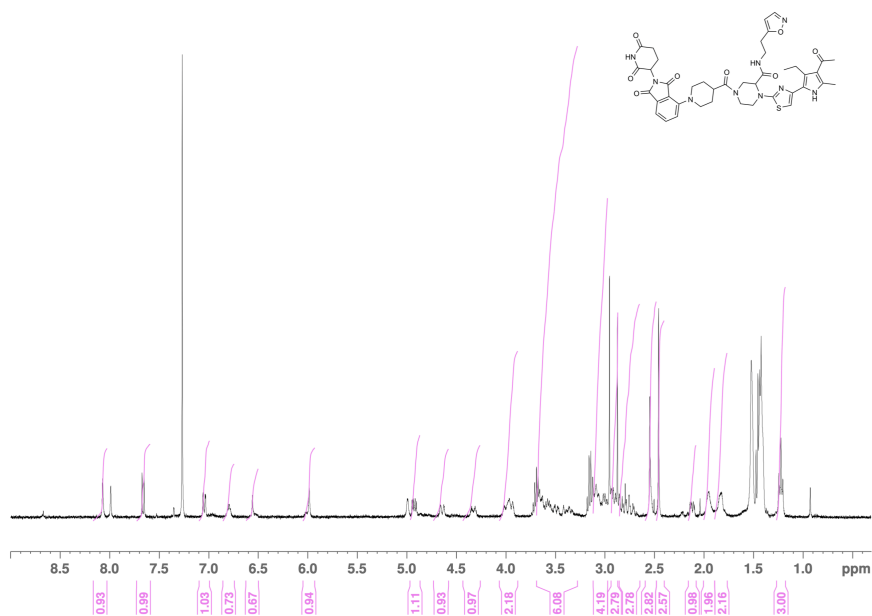
S 8.2: ¹³C NMR Spectrum of 1a in CD₃OD



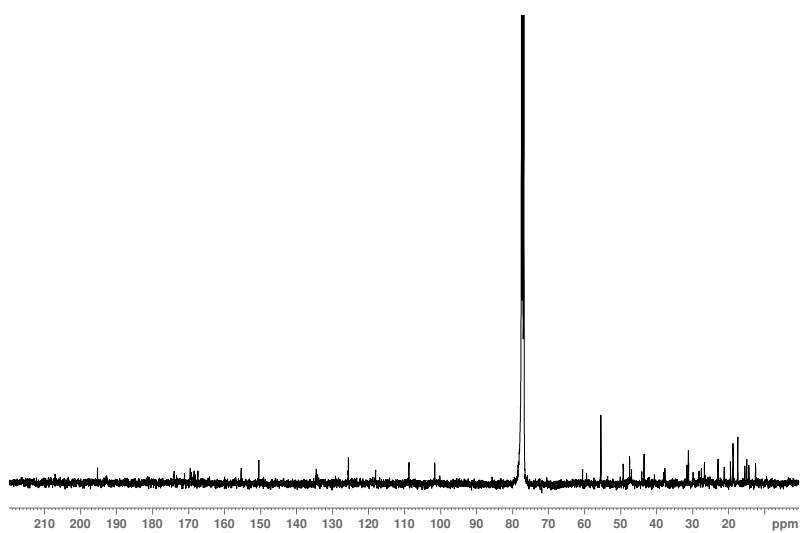
S 8.3: ^1H NMR Spectrum of **2a** in CD₃OD



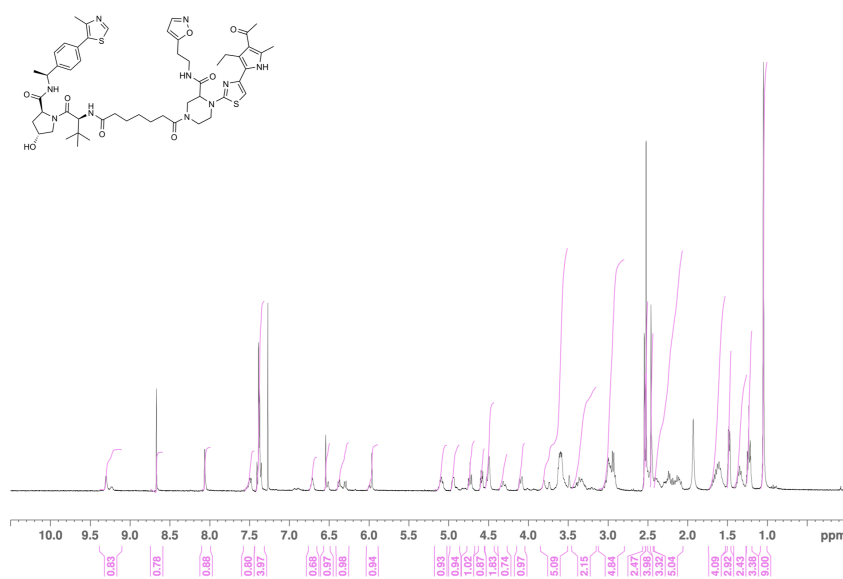
S 8.4: ^{13}C NMR Spectrum of **2a** in CD₃OD



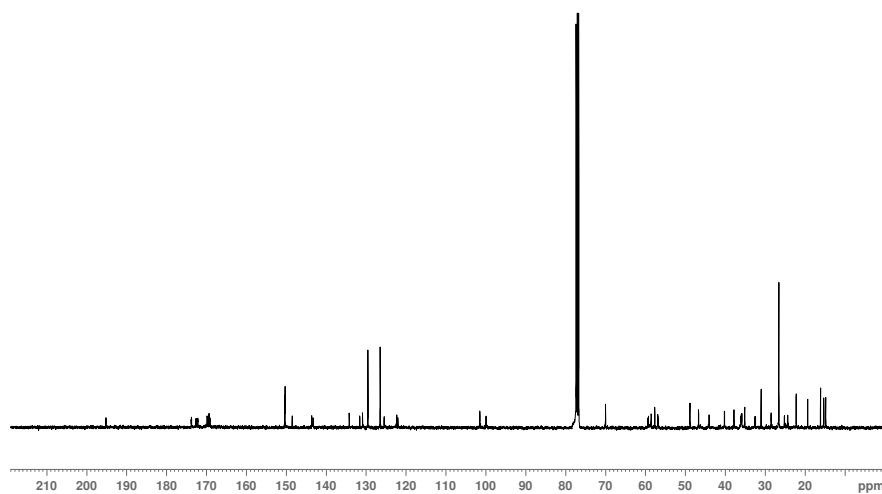
S 8.5: ^1H NMR Spectrum of **3a** in CDCl_3



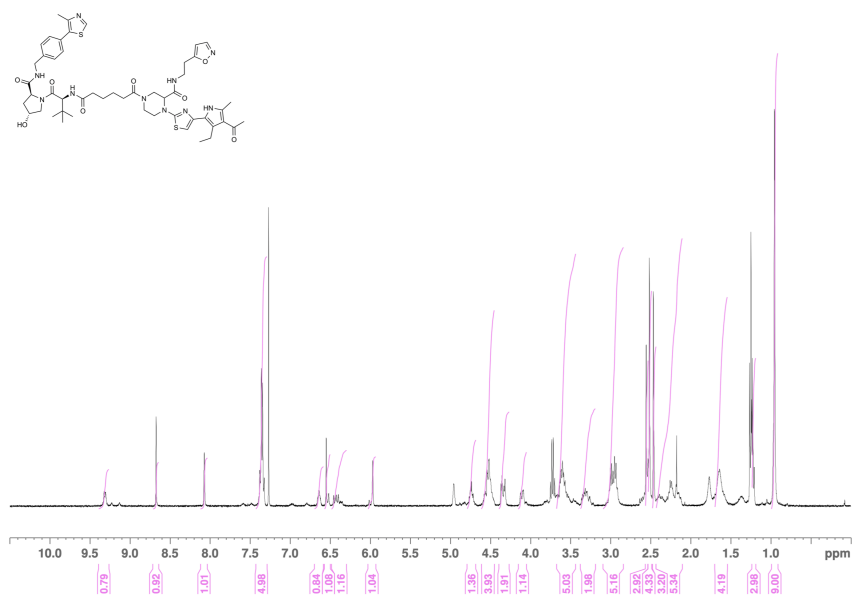
S 8.6: ^{13}C NMR Spectrum of **3a** in CDCl_3



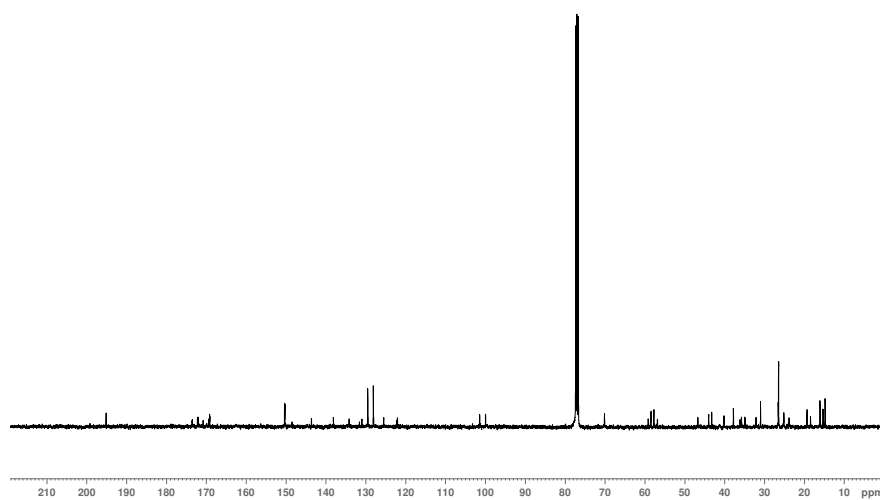
S 8.7: ^1H NMR Spectrum of **1b** in CDCl_3



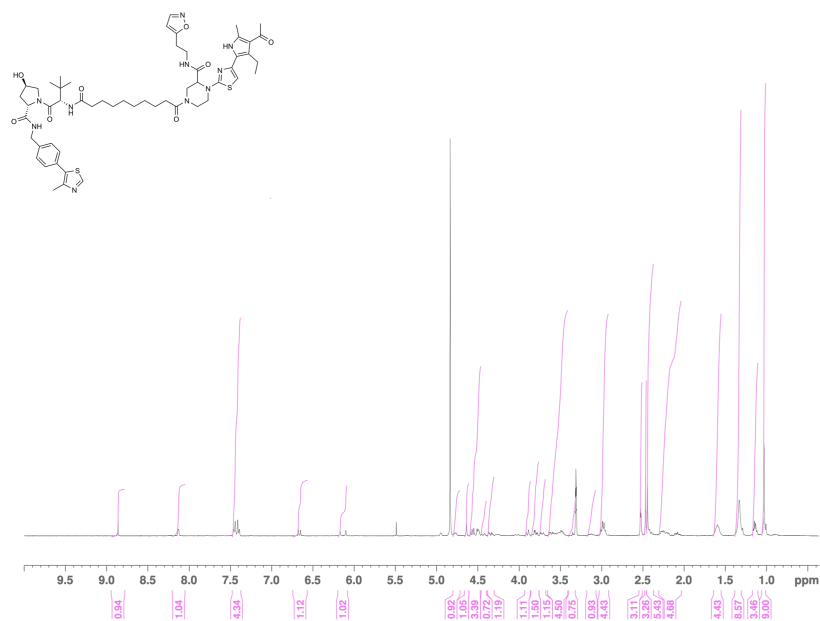
S 8.8: ^{13}C NMR Spectrum of **1b** in CDCl_3



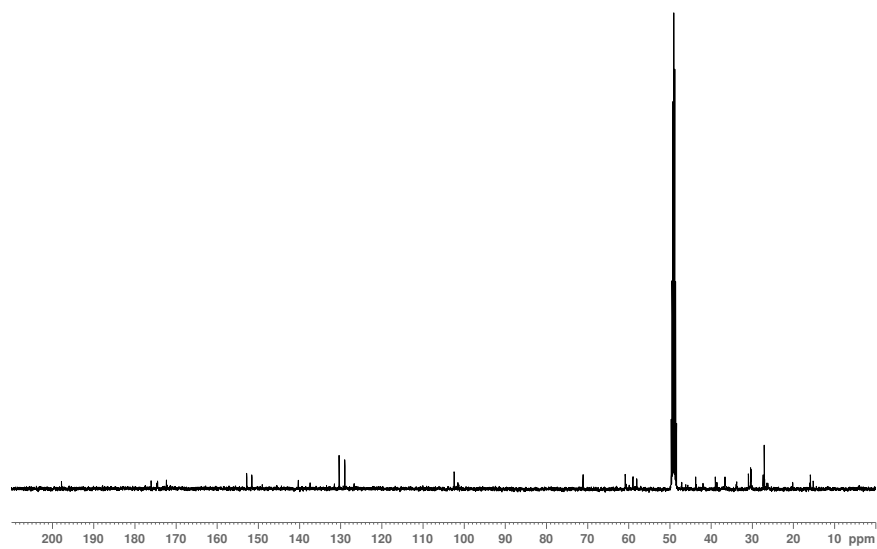
S 8.9: ^1H NMR Spectrum of **2b** in CDCl₃



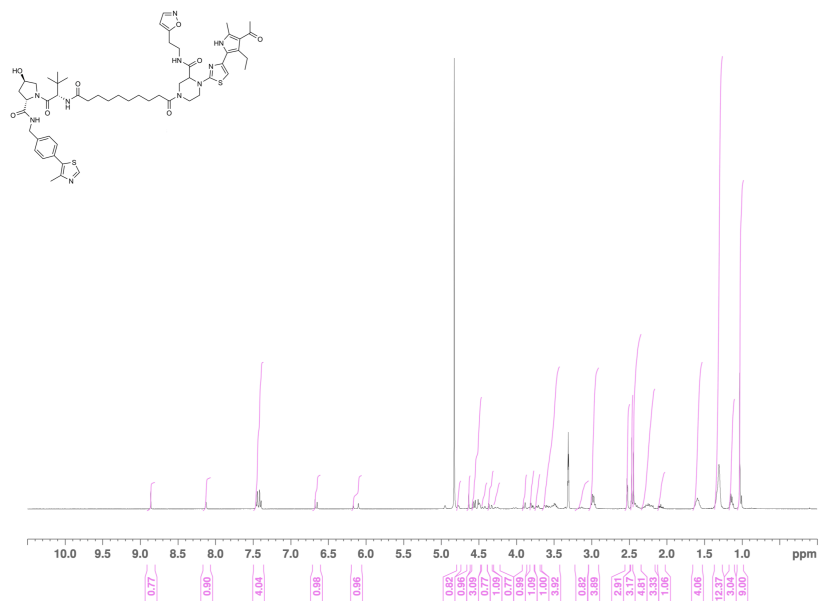
S 8.10: ^{13}C NMR Spectrum of **2b** in CDCl₃



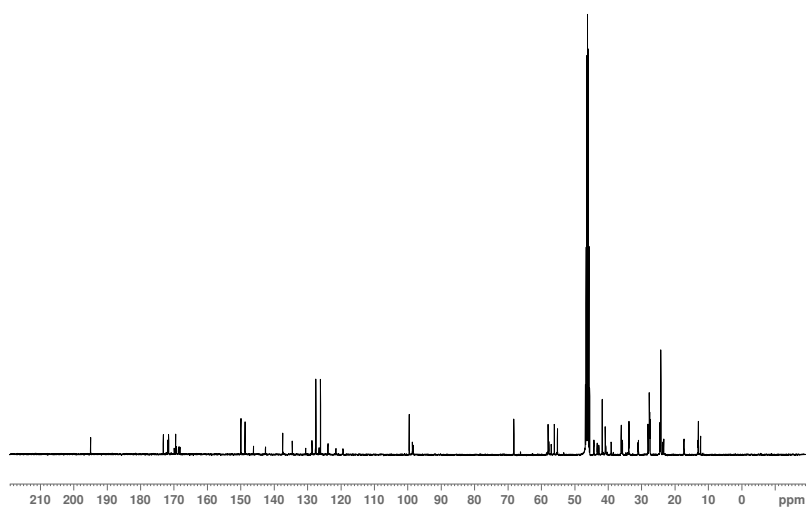
S 8.11: ^1H NMR Spectrum of **3b** in CD_3OD



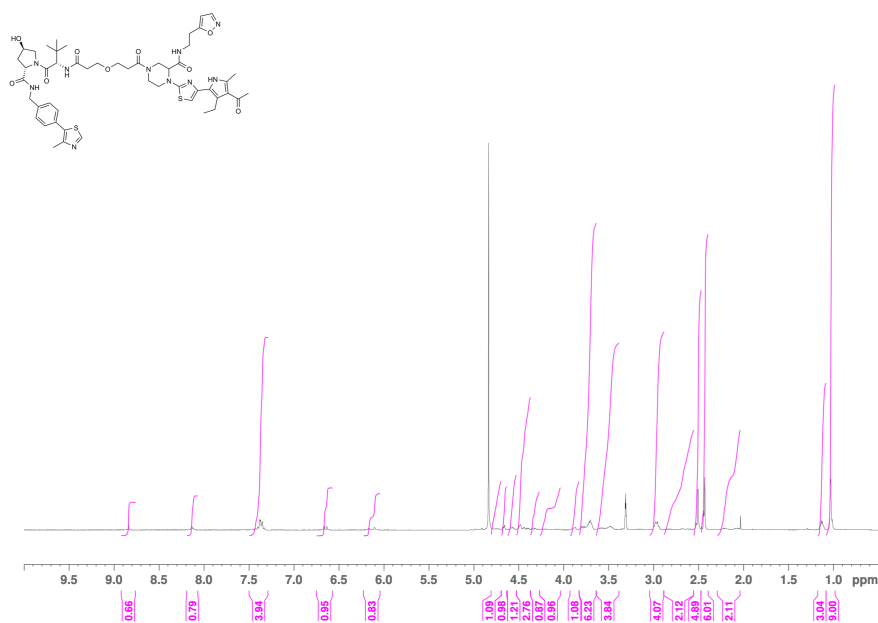
S 8.12: ^{13}C NMR Spectrum of **3b** in CD_3OD



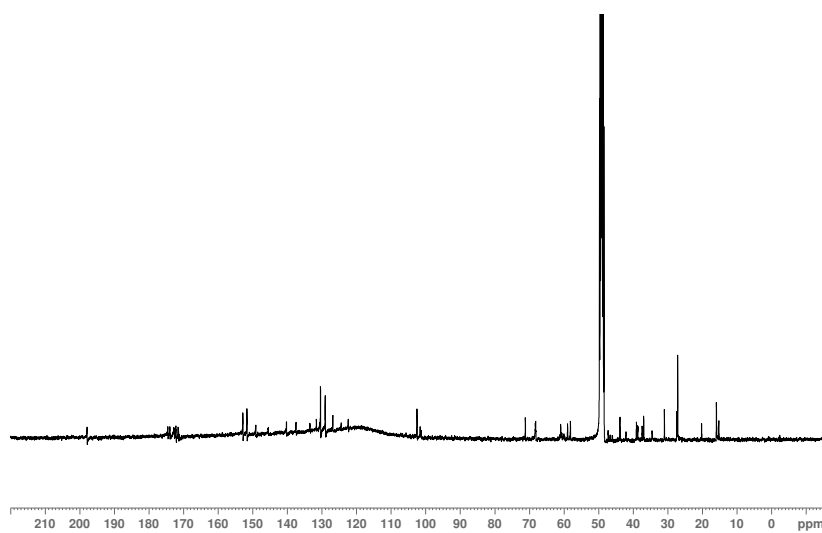
S 8.13: ^1H NMR Spectrum of **4b** in CD₃OD



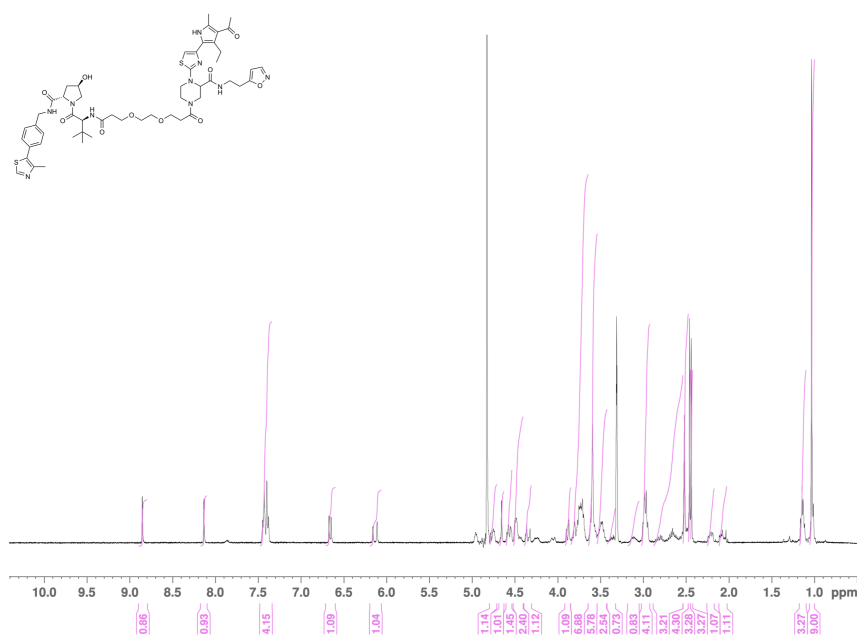
S 8.14: ^{13}C NMR Spectrum of **4b** in CD₃OD



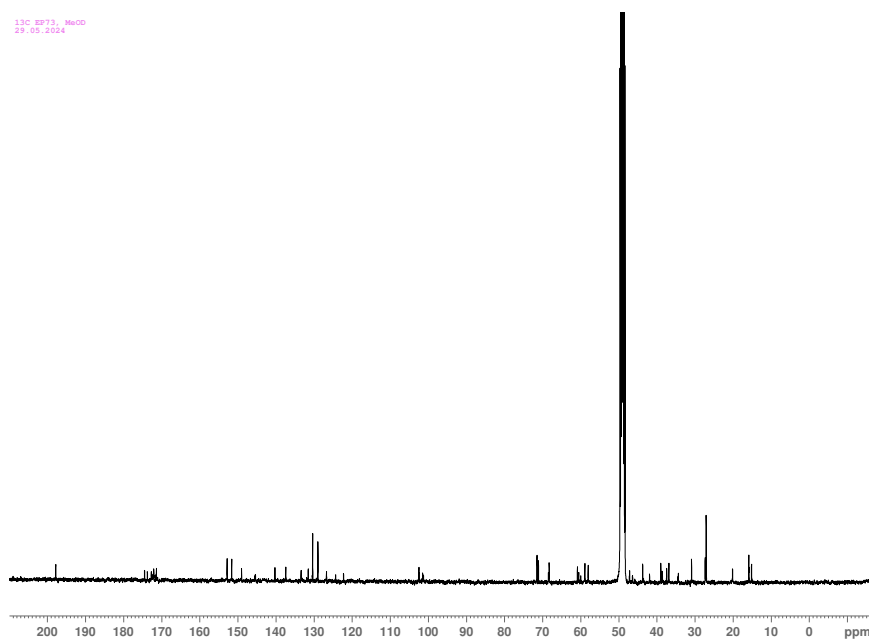
S 8.15: ^1H NMR Spectrum of **5b** in CD_3OD



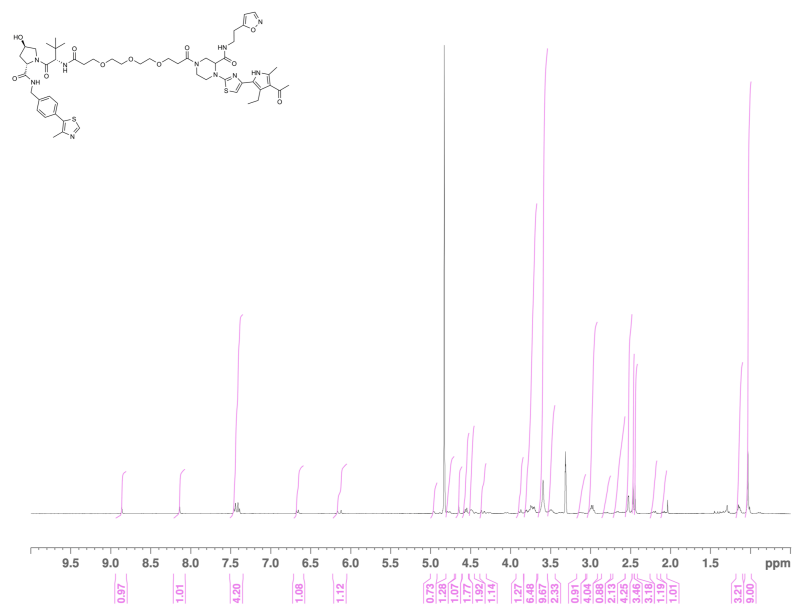
S 8.16: ^{13}C NMR Spectrum of **5b** in CD_3OD



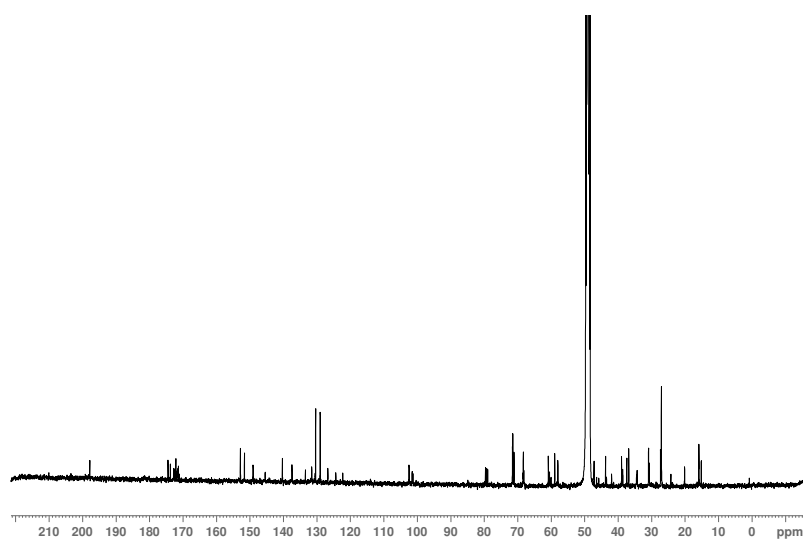
S 8.17: ^1H NMR Spectrum of **6b** in CD_3OD



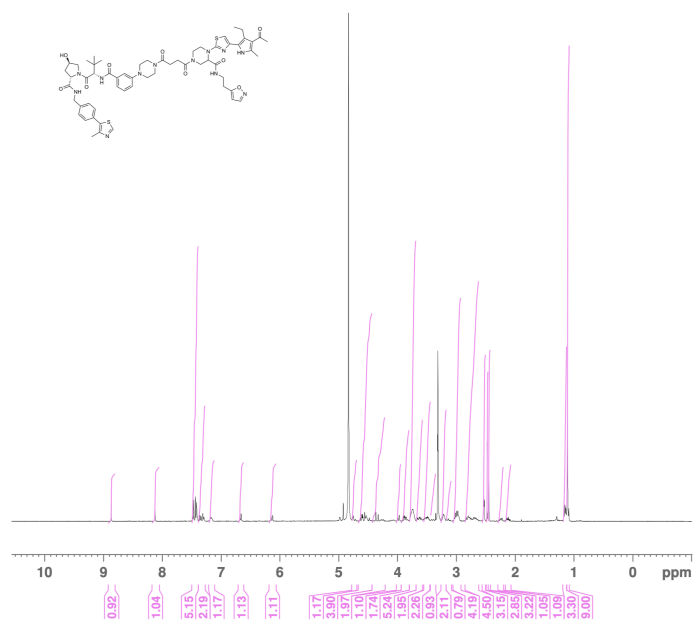
S 8.18: ^{13}C NMR Spectrum of **6b** in CD_3OD



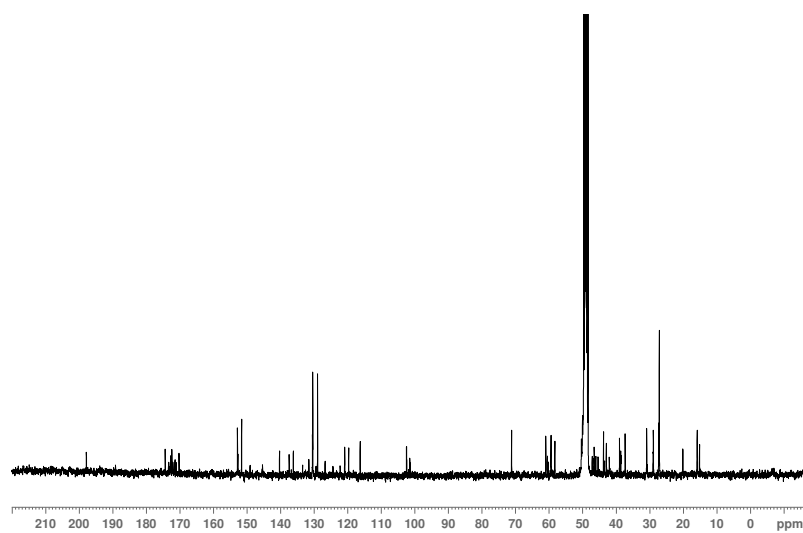
*S 8.19: ^1H NMR Spectrum of **7b** in CD_3OD*



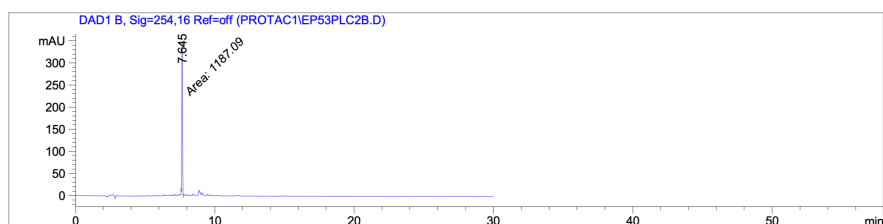
*S 8.20: ^{13}C NMR Spectrum of **7b** in CD_3OD*



S 8.21: ^1H NMR Spectrum of **8b** in CD_3OD



S 8.22: ^{13}C NMR Spectrum of **8b** in CD_3OD

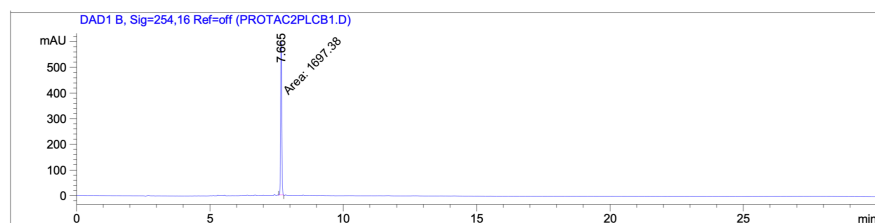


Signal 2: DAD1 B, Sig=254,16 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.645	MM	0.0586	1187.09058	337.38745	100.0000

Totals : 1187.09058 337.38745

S 8.23: HPLC Chromatogram of compound 1a

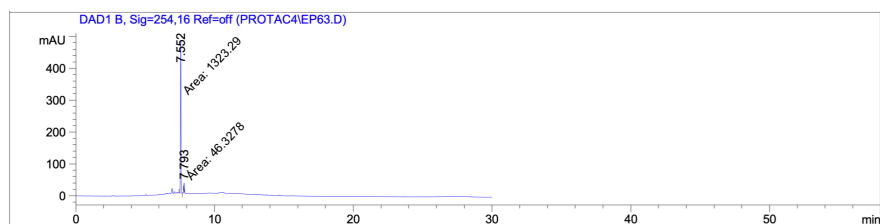


Signal 2: DAD1 B, Sig=254,16 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.665	MM	0.0470	1697.37622	601.89404	100.0000

Totals : 1697.37622 601.89404

S 8.24: HPLC Chromatogram of compound 2a

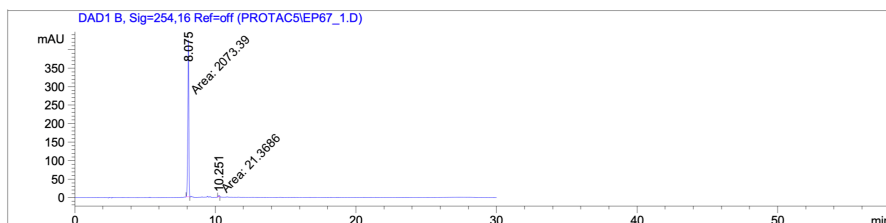


Signal 2: DAD1 B, Sig=254,16 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.552	MM	0.0460	1323.28882	479.92560	96.6175
2	7.793	MM	0.0322	46.32781	23.95266	3.3825

Totals : 1369.61663 503.87826

S 8.25: HPLC Chromatogram of compound 3a

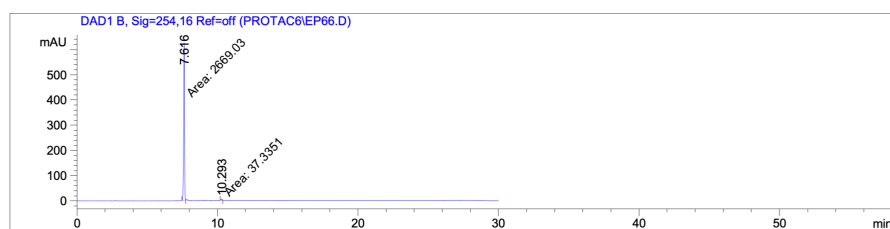


Signal 2: DAD1 B, Sig=254,16 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	8.075	MM	0.0811	2073.38965	425.97147	98.9799
2	10.251	MM	0.0915	21.36862	3.89248	1.0201

Totals : 2094.75827 429.86394

S 8.26: HPLC Chromatogram of compound 1b

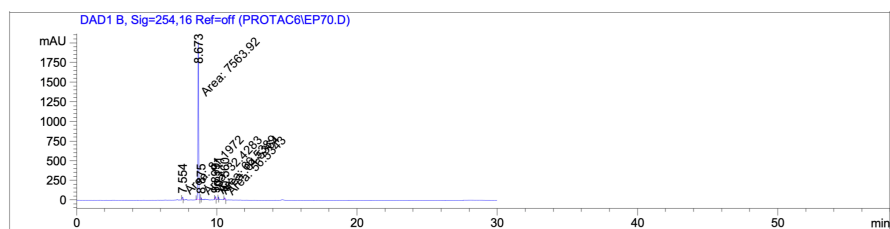


Signal 2: DAD1 B, Sig=254,16 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.616	MM	0.0710	2669.03052	626.92584	98.6205
2	10.293	MM	0.1275	37.33508	4.87894	1.3795

Totals : 2706.36560 631.80478

S 8.27: HPLC Chromatogram of compound 2b

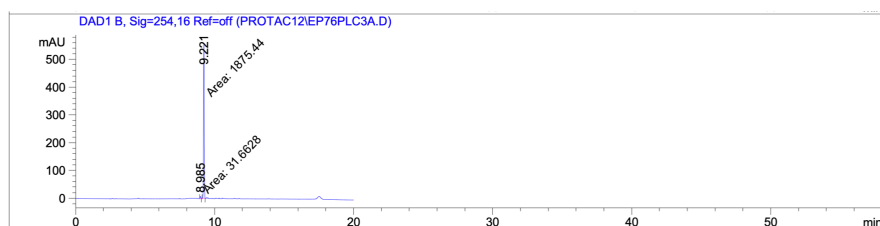


Signal 2: DAD1 B, Sig=254,16 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.554	MM	0.0517	81.19724	26.18887	1.0324
2	8.673	MM	0.0625	7563.91650	2017.84814	96.1700
3	8.875	MM	0.0342	32.42833	15.82626	0.4123
4	9.899	MM	0.0406	66.53886	27.34435	0.8460
5	10.111	MM	0.0432	64.53643	24.88517	0.8205
6	10.560	MM	0.0490	56.53433	19.22416	0.7188

Totals : 7865.15170 2131.31694

S 8.28: HPLC Chromatogram of compound 3b

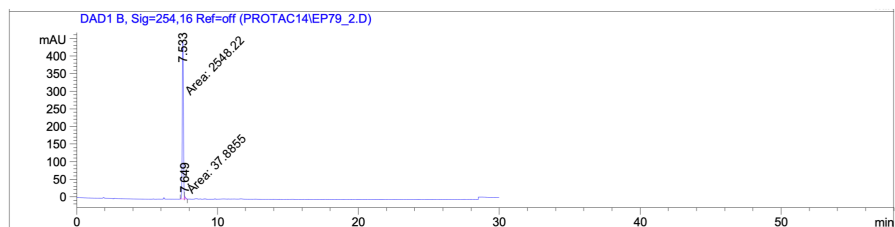


Signal 2: DAD1 B, Sig=254,16 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	8.985	MM	0.0584	31.66278	9.03510	1.6603
2	9.221	MM	0.0555	1875.43921	563.13416	98.3397

Totals : 1907.10199 572.16926

S 8.29: HPLC Chromatogram of compound 4b

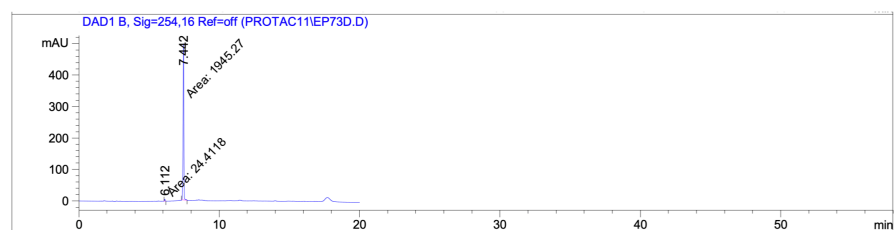


Signal 2: DAD1 B, Sig=254,16 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.533	MF	0.0942	2548.22485	450.89758	98.5350
2	7.649	FM	0.0809	37.88554	7.80638	1.4650

Totals : 2586.11040 458.70396

S 8.30: HPLC Chromatogram of compound 5b

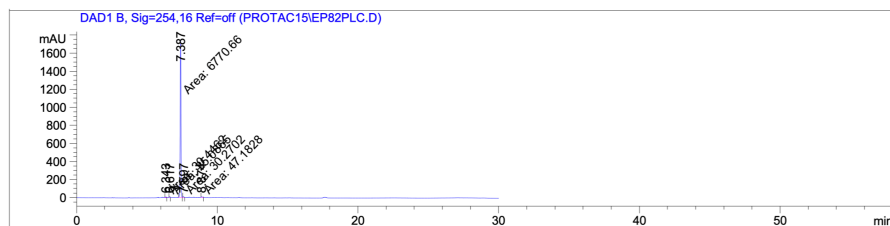


Signal 2: DAD1 B, Sig=254,16 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	6.112	MM	0.0564	24.41183	7.21346	1.2394
2	7.442	MM	0.0649	1945.26660	499.39252	98.7606

Totals : 1969.67843 506.60598

S 8.31: HPLC Chromatogram of compound 6b

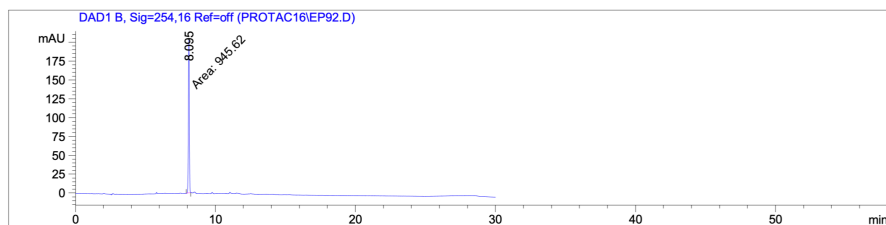


Signal 2: DAD1 B, Sig=254,16 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	6.343	MM	0.0592	30.44621	8.57218	0.4410
2	6.617	MM	0.0442	25.08650	9.46721	0.3634
3	7.387	MM	0.0643	6770.66260	1755.08215	98.0737
4	7.597	MM	0.0557	30.27021	9.05163	0.4385
5	8.874	MM	0.0946	47.18283	8.31110	0.6834

Totals : 6903.64835 1790.48426

S 8.32: HPLC Chromatogram of compound 7b



Signal 2: DAD1 B, Sig=254,16 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	8.095	MM	0.0770	945.62012	204.66960	100.0000

Totals : 945.62012 204.66960

S 8.33: HPLC Chromatogram of compound 8b

La borsa di dottorato cofinanziata con risorse del
Programma Operativo Nazionale Ricerca e Innovazione 2014-2020 (CCI 2014IT16M2OP005),
risorse Fondo Sociale Europeo REACT-EU, Azione I.1 “Dottorati Innovativi con caratterizzazione industriale”, Azione
IV.4 “Dottorati e contratti di ricerca su tematiche dell’innovazione” e Azione IV.5 “Dottorati su tematiche Green”



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